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UNIVERSITY OF ALBERTA

The Mechanism of Apolipoprotein Mediated Cholesterol Efflux

by

© Qianqian Li

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE**.

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UNIVERSITY OF ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled THE MECHANISM OF APOLIPOPROTEIN MEDIATED CHOLESTEROL EFFLUX submitted by Qianqian Li in partial fulfillment of the requirements for the degree of Master of Science.

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Dated: June 17, 1994

Dedication to:

My parents, Li Baolin and Chen Qiaoline
My husband Wang Jianjun

For their love, support and patience

ABSTRACT

The mechanism of apolipoprotein-mediated cholesterol efflux from cells has been studied. Different extracellular cholesterol acceptors (lipid free apolipoproteins, human HDL, LDL, and lipid microemulsion) are used to detect cholesterol efflux through different pathways. Phospholipid efflux is also examined at the same time to investigate the lipid ratio in the efflux particles. The protein-free lipid microemulsion is used as a cholesterol acceptor to demonstrate the lipid efflux through the nonspecific diffusion pathway, while lipid-free apoA-I is employed to detect the specific pathway potentially involving the cellular interaction site.

Results show that from nonspecific pathway phospholipid efflux is slower than cholesterol efflux probably because that the desorption rate of phospholipids is slower than that of cholesterol. In contrast, in apoA-I-mediated lipid efflux, phospholipid efflux is faster than that of cholesterol. Therefore, the overall contribution of each pathway (nonspecific diffusion and free apolipoprotein-mediated pathway) to cholesterol efflux can be predicted by comparing the ratio of phospholipid/cholesterol in the efflux among microemusions, HDL, and apolipoprotein. The function of cellular surface protein is also examined by trypsin treatment of surface proteins. It is found that the apolipoprotein-mediated cholesterol efflux is completely abolished from the trypsin-treated macrophages, suggesting that cholesterol efflux mediated by this mechanism is related to trypsin-sensitive membrane component.

Different cell lines are used to investigate cell specificity in apolipoprotein-mediated cholesterol efflux. Results show that smooth muscle cells resist apolipoprotein-mediated cholesterol efflux but not phospholipid efflux. Further studies demonstrate that the combined effects of Monocyte Colony-stimulating Factor (MCSF), Platelet-derived Growth Factor (PDGF), and phorbol myristate acetate (PMA) enhance cholesterol efflux from smooth muscle cells by 2-3 times without stimulation of phospholipid efflux. The effect is not observed in macrophages. This may suggest that the

enhancement of cholesterol efflux is mediated by cellular specific mechanism involving only cholesterol efflux rather than phospholipid efflux.

We conclude that apolipoprotein-mediated cholesterol efflux is cell-line specific, trypsin-sensitive, and cellularly regulated process.

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ABBREVIATIONS

ACAT	acyl-CoA cholesterol acyltransferase
apo	apolipoprotein
BSA	bovine serum albumin
CAD	coronary artery disease
CE	cholesterol ester
CETP	cholesterol ester transfer protein
CHO	Chines hamster ovary-derived cell
CM	chylomicron
DTNB	dithionitrobenzoic acid
ER	endoplasmic reticulum
FBS	fetal calf serum
FC	free cholesterol
HDL	high density lipoprotein
HMG	3-hydroxyl-3-methylglutaryl
LCAT	plasma lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
MCSF	monocyte colony-stimulating factor
ME	microemulsion
MEM	minimum essential medium
nCEH	neutral cholesterol ester hydrolase
NEM	N-ethylmaleimide
ox LDL	oxidized LDL
PBS	phosphate buffered saline
PC	phosphatidylcholine
PDGF	platelet-derived growth factor
PKC	protein kinase C
PMA	phorbol myristate acetate
SMC	smooth muscle cell
TG	triacylglycerol
TLC	thin layer chromatography
VLDL	very low density lipoprotein

Chapter I.

Introduction

1. Lipoproteins and apolipoproteins

The plasma lipoproteins are soluble complexes of lipids and specialized proteins (apolipoproteins). They are composed of an outer shell and an inner hydrophobic core. The outer shell contains polar phospholipid, free cholesterol and apolipoproteins; the core is composed of triacylglycerols and cholesterol esters. The main function of these lipoproteins is to deliver lipids to peripheral tissues. Four classes of lipoproteins are defined according to their densities: chylomicron (CM) ($d<1.00$ g/ml), very low density lipoprotein (VLDL) ($d=1.006-1.02$ g/ml), low density lipoprotein (LDL) ($d=1.02-1.063$ g/ml), and high density lipoprotein (HDL) ($d=1.063-1.21$ g/ml). CM and VLDL function in the transport of triacylglycerols while LDL and HDL are related to cholesterol metabolism.

Apolipoproteins are the lipid-free protein components of the plasma lipoproteins obtained by treating intact lipoproteins with organic solvents, detergents, or chaotropic agents. Current knowledge of their distribution, molecular weights, polymorphism, origins, concentration and functions is summarized in Table.1. ApoA-I, A-II, A-IV, C-I, C-II, C-III, and E all contain amphipathic a-helices. Most apolipoproteins, which are located primarily on the lipoprotein surface, readily exchange among lipoproteins. Unlike all the other apolipoproteins, however, apo B cannot be transferred between lipoproteins.

1.1. Structure and function of apolipoproteins

1.1.1. Apo B

Apo B is one of the largest proteins isolated and characterized (500 kD). Apo B appears critical to LDL receptor- mediated uptake of LDL and to the assembly of apo B containing lipoproteins. Chemical modifications of its arginine or lysine residues can abolish the binding of apo B with LDL receptor and result in the uptake of the modified LDL by the scavenger receptor or oxidized-LDL receptor, inducing accumulation of cholesterol in macrophages. This is relevant to the mechanism for the formation of foam cells. Unlike all other apolipoproteins, apo B cannot undergo exchange between lipoproteins. Therefore apo B may also play an important role in structural stability of lipoproteins.

1.1.2. Other apolipoproteins

The other apolipoproteins which associate with lipoproteins all have the amphipathic a-helix structure as their common feature (Segrest, et al. 1974). There are two distinct functions of amphipathic α-helices in apolipoproteins: (A) self-association via hydrophobic sequences allows the protein to exist in solution as a water soluble complex, (B) association with the membrane bilayer via proteins containing amphipathic α-helices allows the proteins to bind reversiblely to the membrane (Davis, 1991). Therefore the properties of α-helices in apolipoproteins allow apolipoproteins to exist in different forms: an unassociated lipid-free form, a lipid-associated form, or a membrane-associated form. These characteristics also allow α-helix containing apolipoproteins to exchange freely among different lipoproteins.

These apolipoproteins play very important roles in lipoprotein metabolism. ApoA-I is 28 KD protein and major protein component of HDL. More than 90% of plasma apoA-I is associated with HDL, less than 1% is present on VLDL. HDL-associated apoA-I is a potent activator of plasma lecithin:cholesterol acyltransferase (LCAT) which is important for HDL metabolism. In transgenic mice, overexpress of apoA-I can protect against atherosclerosis (Breslow, 1993). ApoA-II is also a major constituent of human HDL. The specific role of apoA-II has not been identified yet. However, overexpress of apoA-II does not show antiatherogenic effect. ApoC-I makes up approximately 10% of VLDL protein and 2% of HDL protein. It can also activate LCAT. ApoC-II is a 8.8 KD protein that associates with triacylglycerol-rich lipoprotein and activates lipoprotein lipase, facilitating rapid clearance of triacylglycerol-rich lipoproteins from blood. ApoC-III accounts for 50% of the protein in VLDL. In vitro, apoC-III can diminish lipoprotein lipase activity. ApoE comprises 10-20% of VLDL protein. ApoE has high affinity for LDL receptor and mediates the uptake of VLDL and remnants. Since these small apolipoproteins can exchange among lipoproteins, the ratio of these proteins on lipoprotein surfaces also regulates the metabolism of lipoproteins. Additionally, the lipid free apolipoproteins, such as apoA-I, can interact with cell membranes to facilitate lipid efflux from cells (Hara and Yokoyama 1991). This pathway may play a role in the regression and prevention of atherosclerosis and generation of new HDL particles.

1.2. Structure and function of lipoproteins

1.2.1. Triacylglycerol-rich lipoproteins

CM and VLDL are of intestinal and hepatic origin respectively. Both are triacylglycerol-rich lipoproteins, consisting of a monolayer surface of phospholipids and free cholesterol as well as a large hydrophobic core of triacylglycerol and cholesterol ester. In addition to lipids, apo B and several minor apolipoproteins associate with the monolayer. Apo B plays a key role in the intracellular assembly of these lipoproteins for secretion. Apo C-II is required for activation of lipoprotein lipase and apo E serves as a ligand for tissue targeting. The triacylglycerol in these lipoproteins is rapidly hydrolyzed by lipoprotein lipase (which anchors to endothelial cells) and delivered to tissues such as adipose and muscle.

1.2.2. Low density lipoprotein (LDL)

LDL is primarily derived from lipolysis of VLDL by lipoprotein lipase. Each LDL contains an apo B-100 on the surface and cholesterol ester as the major core lipid. Apo B mediates the uptake of LDL through the LDL receptor and transports cholesterol into tissues. Modified LDL (via oxidation) cannot be recognized by the LDL receptor, while it can be taken up through the high affinity binding site (scavenger receptor) on macrophages. This leads to accumulation of cholesterol in the cells of the arterial wall and formation of foam cells which are the major cells in atherosclerosis lesions.

1.2.3. High density lipoprotein (HDL)

1.2.3.1. HDL synthesis and secretion:

The lipids and apolipoproteins of HDL are exchangeable. This leads to the existence of different HDL particles in plasma in terms of apolipoprotein composition, size and function. The formation process of HDL is not fully understood. The experiments using cultured cells and perfused organs show that discoidal HDL may be the nascent HDL, containing only a bilayer of phospholipids, free cholesterol and apoA-I without core lipids (Hamilton, et al. 1976). Discoidal HDL is an excellent substrate of LCAT which is activated by apoA-I on HDL and catalyzes cholesterol esterification in plasma. As cholesterol is esterified in HDL, cholesterol moves from the surface into the core. The accumulation of cholesterol ester in the core changes the shape of HDL from disk to mature sphere formation. However, the assembly of discoidal HDL remains to be identified. Discoidal HDL cannot be isolated from Golgi of hepatocytes, which is the major site of lipoprotein secretion, suggesting assembly of HDL may be not intracellular. In vitro experiment, it is found that the major apolipoproteins of HDL (apoA-I and apoE) can be synthesized and secreted by cultured hepatocytes and macrophages (Thrift, et al. 1986; Davis, et al. 1989). Lipid and apolipoprotein complexes are formed when these apolipoproteins are incubated with serum, suggesting that HDL is assembled extracellularly. Extracellular assembly of HDL may play an important role in cellular lipid removal.

1.2.3.2. Function of HDL

HDL has been considered as a potent factor in the prevention of atherosclerosis. The supporting evidence includes: 1) HDL is an acceptor of cholesterol from the nonhepatic cells in vitro (Oram, et al. 1990); 2) an increase in HDL cholesterol is associated with a reduced risk of coronary artery disease (CAD) (Gordon, et al. 1977); 3) high or low plasma levels of HDL in genetic mutation are associated with resistance or earlier development of CAD, respectively (Norum, et al. 1982; Ordovas, et al. 1989). It is believed that this is mainly because HDL participates in reverse cholesterol transport, the process by which cholesterol is transported from peripheral cells to the liver. This pathway is believed to be the major route for removal of exchangeable cholesterol deposited in nonhepatic tissues. However, the mechanism of reverse cholesterol transport has not been fully understood yet. There are several hypotheses for cellular cholesterol efflux (the initial step of reverse cholesterol transport): nonspecific diffusion mechanism and HDL-binding protein mediated mechanism. The former suggests that HDL-mediated cholesterol efflux is a nonspecific exchange process (William, et al. 1991). The latter suggests that cholesterol efflux is mediated by the HDL-binding protein which promotes cholesterol translocation from intracellular pool to cell membrane pool (Oram, et al 1991). Data from Yokoyama's lab show that new HDL-like particles are generated when low concentration (1/500 plasma) of free apolipoproteins are incubated with cholesterol-loaded macrophages and fibroblasts. This suggests that small amounts of apolipoprotein dissociated from lipoproteins may interact with cell membranes and generate HDL-like particles extracellularly. This pathway may contribute not only to HDL generation but also to cholesterol removal from foam cells (Hara and Yokoyama, 1991).

In addition to the role in reverse cholesterol transport, HDL is proposed to have other roles in preventing atherosclerosis (review by Badman, et al. 1992) : 1) it acts as an antioxidant preventing LDL oxidation, 2) it reduces platelet aggregatability, 3) apo A-I, the major protein of HDL, promotes fibrinolysis, 4) it inhibits LDL uptake by LDL-receptor in peripheral cells, 5) HDL or apo A-I protect LDL aggregation. However, the beneficial role of HDL in atherosclerosis remains to be established.

1.2.3.3. Apolipoproteins on HDL

The major apolipoproteins associated with HDL are apoA-I, apoA-II, apoE and apoCs. ApoA-I is synthesized in the liver and intestine; apoA-II originates from the liver; apoE is synthesized by many tissues including the liver and macrophages. The rate of synthesis of apoA-I is regulated under metabolic control while apo E is regulated by fasting (in the liver) and by cholesterol levels (in macrophages). HDL apolipoproteins are exchangeable among lipoproteins. ApoA-I moves off and on HDL in response to metabolic changes. This allows free apoA-I to interact with the cell membrane and generate new HDL-like particles by mediating cholesterol efflux from the cell (Hara, et al. 1991). Compared to apoA-I, apoA-II cannot transfer readily between native HDL particles because apoA-II has higher affinity for HDL. Therefore, by regulating the rate of apoA-I and apoA-II synthesis, the plasma HDL level can be regulated.

2. Cholesterol transfer among lipoproteins

The plasma lipoproteins are continuously remodeled during their transport through the plasma compartment, owing to the action of lipid-metabolizing enzymes and lipid transfer processes. LCAT is responsible for the esterification of lipoprotein cholesterol, and controls indirectly the levels of free and esterified cholesterol in cells (Glomset, 1972). Cholesterol ester transfer protein (CETP) promotes the transfer of LCAT-derived cholesterol ester between lipoproteins.

2.1. Function of LCAT

LCAT is an extracellular enzyme secreted by the liver. LCAT transfers an acyl chain from phosphatidylcholine (PC) to cholesterol, producing lysoPCs and cholesterol ester. In this reaction, apoA-I, the major protein of HDL, is a cofactor. Therefore the discoidal HDL containing lecithin, free cholesterol and apoA-I can be the major substrate of LCAT. After cholesterol on the surface of discoidal HDL is esterified, it migrates from the surface into the core of the particle. This change alters the shape of HDL from disk to sphere, forming mature HDL particles. LCAT also esterifies cholesterol from circulating lipoproteins, but mainly on HDL particles containing apo A-I/A-II (Jonas, et al. 1887). This reaction on HDL generates the gradient of unesterified cholesterol between cell membranes and HDL particles. This promotes cholesterol transfer from cell membrane to HDL and contributes to reverse cholesterol transport. By converting the excess unesterified surface cholesterol and lecithin into cholesterol ester, LCAT also facilitates the reaction of lipoprotein lipase and influences the metabolism of triacylglycerol-rich lipoproteins (Jonas, 1991).

2.2. The function of cholesterol ester transfer protein (CETP)

CETP facilitates the movement of cholesterol ester, triacylglycerol, and other nonpolar lipids between plasma lipoproteins. Physiologically, the main effect of CETP may promote the transfer of LCAT-derived cholesteryl ester from HDL into VLDL and LDL, redistributing cholesterol ester among lipoproteins and facilitating the reaction of LCAT. Differing from LCAT that functions as an enzyme to catalyze a irreversible reaction, CETP is a carrier protein that distributes cholesterol ester among lipoproteins. A net transfer of cholesteryl ester occurs only when a gradient exists. Two possible mechanisms have been proposed for the reaction mechanism: 1) CETP serves as a soluble carrier of lipid, increasing the lipid diffusion rate down its concentration gradient between different lipoproteins (Barter, et al. 1980); 2) CETP acts as a molecular glue extending the association between lipoprotein particles (Ihm, et al. 1982). However, the physiological function and reaction mechanism of CETP are not fully understood yet.

3. Cholesterol transport between lipoproteins and cells

All animal cells require cholesterol to support membrane biosynthesis. Cells can utilize either endogenous or exogenous sources of cholesterol. Endogenous cholesterol can be derived in cells in two ways: by synthesis or by mobilization of cholesterol from cholesterol ester storage pools. Exogenous cholesterol comes from circulating lipoproteins. Low-density lipoprotein particles are the primary carrier of cholesterol in blood plasma. Cells have several mechanisms to maintain unesterified cholesterol levels. Exogenous cholesterol can be delivered to macrophages through the LDL receptor (Brown, et al. 1986). In addition to the LDL receptor, macrophages have at least two other classes of receptors which recognize modified LDL. The scavenger receptor recognizes both acetyl LDL and oxidized-LDL (oxLDL) (Goldstein, et al. 1979; Henriksen, et al. 1983; Kodama, et al. 1990), and the oxidized LDL receptor (oxLDL receptor) recognizes only oxLDL (Parthasarathy, et al. 1986; Koo, et al. 1986).

3.1. LDL-receptor mediated cholesterol uptake

The LDL receptor is synthesized in the endoplasmic reticulum (ER) and transferred to the cell surface. On the membrane, the receptors form coated pits. After the receptor binds to apoB and apoE containing lipoproteins, the lipoproteins are internalized and delivered to lysosomes where the receptors migrate back to the cell membrane and the cholesterol is used for biosynthesis and regulatory functions. The regulatory steps include: inhibition of LDL receptor production, suppression of 3-hydroxyl-3-methylglutaryl (HMG) coenzyme A reductase (a key enzyme in cholesterol biosynthesis), and stimulation of acyl-CoA cholesterol acyltransferase (ACAT) which catalyzes cholesterol esterification.

3.2. Uptake of modified LDL

LDL particles can be modified during their prolonged residency in both intima and in hypercholesterolemic plasma. The modified LDL appears to be recognized by specific sites on macrophages (oxLDL receptor and scavenger receptor) (Fogelman, 1988). Recently, scavenger receptors have been found on smooth muscle cells also (Inaba, et al. 1992). The scavenger and oxLDL receptors are not down regulated by the cellular cholesterol level. As a result, macrophages and smooth muscle cells exposed to modified LDL tend to accumulate a large amount of cholesterol and eventually become foam cells which are the hallmark of atherosclerosis.

4. Foam cell and atherosclerosis

The early stage of atherosclerosis is the so-called 'fatty streak', mainly composed of lipid-rich macrophages and lymphocytes. The fatty streak precedes development of intermediate lesions, consisting of macrophages and smooth muscle cells. In the advancing stage, fibrous plaques are formed as a dense cap which contains connective tissue and smooth muscle cells. Both macrophages and smooth muscle cells can accumulate lipid and form foam cells within the lesions of atherosclerosis. The foam cells in the lesions have a profound influence on atherosclerosis (Ross, 1993).

4.1. Macrophages

The roles of macrophages in atherosclerosis are: (1) accumulation of large amount of cholesterol through the uptake of oxLDL by the scavenger and the oxLDL receptors, (2) oxidation of LDL through lipoxygease, exacerbating the atherogenic process, (3) production of growth factors, such as Platelet-derived Growth Factor (PDGF) (Shimokado, et al. 1985), leading to proliferation of cells in the arterial wall. Therefore, macrophages are very important in the generation and development of lesions not only because of their scavenger role but also because of the growth stimulatory functions.

4.2. Smooth muscle cells

There are two phenotypes of smooth muscle cells, contractile and synthetic phenotype. In the lesions, they transform into the synthetic state (Sjolund, et al. 1988) and express growth factor receptors such as PDGF receptors (Shimokada, et al. 1985). Furthermore, they can secrete and release growth stimulators, promoting further proliferation in the lesions (Baird, et al. 1990).

5. Reverse cholesterol transport

Cholesterol accumulated within the cells cannot be further catabolized but must be transferred from peripheral cells to the liver where cholesterol is catabolized to bile acid (Fogelman, et al. 1982). This process is called "reverse cholesterol transport". Reverse cholesterol transport is the only pathway to remove excess cholesterol from peripheral cells. Therefore, this pathway is essential for regression and prevention of atherosclerosis. The pathway involves several key steps in vivo: 1) unesterified cholesterol efflux from cells to cholesterol acceptors with HDL as the major acceptor in vivo (Gordon, et al. 1977); 2) LCAT conversion of cholesterol in the various precursor HDL particles to cholesterol ester (CE) (Jonas, et al. 1987); 3) redistribution of cholesterol among HDL and LDL by the combined actions of LCAT, hepatic lipase and CETP (Garfinkel, 1987; Tall, et al. 1986); 4) hepatocyte uptake of HDL and LDL particles. Since cholesterol efflux from cells is the first step in reverse cholesterol transport, it has been intensively studied recently. Several hypotheses have been established based on different studies.

5.1. Nonspecific exchange/diffusion model

It is well known that unesterified cholesterol undergoes free exchange among lipoproteins, as well as between lipoproteins and cells or artificial membranes without the requirement of free energy (Rothblat, 1982). This process may only be a physical-chemical phenomenon: cholesterol molecules dissolve from cell membranes and diffuse through the aqueous layer around the cell until they collide with an acceptor particle. In this process, the rate-limiting step is the desorption of cholesterol molecules from the cell membrane (Rothblat, et al. 1982). The direction of cholesterol flux is determined by the concentration gradient of cholesterol between the cell membranes and the acceptors. Therefore, it is believed that cholesterol efflux from cells to extracellular acceptor is a surface transfer process which does not require lipoprotein internalization (Bruckdorfer, et al., 1976). HDL-induced cholesterol efflux is consistent with this diffusion mechanism (William, et al. 1991). In this way, HDL-mediated cholesterol efflux is only a simple cholesterol exchange between cells and HDL particles. A net cholesterol efflux only occurs in cholesterol enriched cells, in which the ratio of cholesterol/PC in plasma membrane is higher than that of HDL (Gold, et al. 1990). The mechanism for this process only involves a nonspecific exchange of lipids between HDL particles and cell. Thus the major factors influencing HDL-mediated cholesterol efflux are physiochemical factors such as lipid composition or/and structure of HDL and the plasma membranes. Phospholipid efflux mediated by this mechanism is much slower than that of cholesterol (William, et al. 1991)

5.2. Membrane cholesterol domain model

The nonspecific exchange model cannot explain the differences of cholesterol efflux between different cells therefore the membrane cholesterol domain model has been introduced by the same researchers (Rothblat, 1992; Mahberg, et al. 1992). The central premise of this model is the existence of specific cholesterol domains within the plasma membrane. The packing of cholesterol within these domains is controlled by the strength of the cholesterol interaction with adjacent phospholipid, phospholipid composition, and the presence of membrane proteins. The rate of cholesterol desorption from the membrane is influenced mainly by the strength of interaction between cholesterol and adjacent phospholipid molecules. Therefore, the rate of cholesterol efflux is influenced by the distribution of cholesterol between cholesterol membrane domains. The cholesterol efflux is assumed to be faster from cholesterol-poor domains than that from cholesterol-rich domains because cholesterol is less tightly packed and more likely to undergo desorption from the cholesterol-poor domains. According to the different distribution of cholesterol between the domains, the cholesterol efflux from cells can be assigned to fast, intermediate and slow kinetic pools. Therefore, the different rate of cholesterol efflux from different types of cells may be related to the different cholesterol distribution between the domains. Based on this model, it is found that smooth muscle cells only have slow pools (Mahberg, et al. 1992). However, the role of the membrane cholesterol domains in cholesterol efflux remains to be established.

5.3. HDL-binding protein model

Cholesterol efflux may not involve only nonspecific diffusion but may also involve specific HDL-binding protein. Oram and his coworkers demonstrated the presence of HDL-binding proteins on the cell membranes of various cells including smooth muscle cells and macrophages by ligand blotting experiments (Graham, et al. 1987). Recently, a cellular HDL binding protein has been cloned and expressed (McKnight, et al. 1992). The binding of HDL with the cell surface activates signal transduction pathway that includes protein kinase C (PKC) and cholesterol is translocated from the intracellular pool to the plasma membrane pool by a unknown process; the plasma membrane cholesterol is then removed from cells by extracellular acceptors (Avira, et al. 1989 ; Mendez, et al. 1991; Oram, et al. 1991). However, the same group also showed that cholesterol efflux directly from the plasma membrane is not promoted by the binding of HDL (Oram, et al. 1991). Therefore the real function of these proteins in cholesterol efflux has yet to be identified.

5.4. Free apolipoprotein-mediated lipid efflux

Yokoyama and colleagues have demonstrated that lipid free apolipoproteins (apoAI, AII, E) are capable of interacting with cholesterol-loaded macrophages and fibroblasts, inducing cholesterol efflux (Hara, et al. 1991). This interaction has the following characteristics: 1) It mediates a net cholesterol efflux, therefore it reduces cellular accumulated cholesterol. 2) It requires only very low apoAI concentration to reach its Vmax. 3) It forms particles of apolipoprotein, phospholipid and cholesterol similar in mobility, density, and lipid composition to preβ-HDL, the first acceptor of cellular cholesterol efflux among all plasma lipoproteins (Castro, et al. 1988; Miida, et al. 1990). Based on these characteristics, it is hypothesized that a small amount of apolipoproteins dissociated from HDL particles interact with cells to mediate net cholesterol efflux and generate new HDL-like particles. More recently the preb-HDL particles generated by this reaction from Chines hamster ovary (CHO) cells have been observed with electron microscopy (Forte, et al. 1993). Therefore, this pathway may be a major pathway for cholesterol efflux and for HDL formation.

6. Present studies

The present study focuses on the mechanism of apolipoprotein-mediated cholesterol and phospholipid efflux from macrophages and smooth muscle cells. Different acceptors (lipid free apolipoproteins, HDL particles, LDL particles and lipid microemulsion) were used to investigate the possible mechanism involved in this pathway. The protein-free lipid microemulsion was used as a lipid acceptor to demonstrate the lipid efflux through the nonspecific diffusion pathway, while lipid-free apoA-I was employed to detect the pathway potentially involving cellular interaction sites. Results show that phospholipid efflux is slower than that of cholesterol efflux in microemulsion-mediated lipid efflux since the desorption rate of cholesterol is faster than that of phospholipids. In contrast, apoA-I mediates faster phospholipid efflux than that of cholesterol. Therefore, the overall contribution of each pathway can be predicted by comparing the ratio of phospholipid/cholesterol in lipid efflux among microemulsions, HDL, and apolipoproteins.

On the other hand, the function of cellular surface protein was examined by modification of the surface by trypsin treatment. It was found that free apoA-I-mediated cholesterol efflux was completely inhibited by trypsin-treatment of macrophages, suggesting that the apolipoprotein mediated cholesterol efflux involved trypsin-sensitive component of the membrane. Cellular specificity of the apolipoprotein-mediated cholesterol efflux was investigated using different cell lines. Results show that smooth muscle cells resist apolipoprotein-mediated cholesterol efflux but not phospholipid efflux.

In further studies, it was also demonstrated that the combined effects of Monocyte Colony-stimulating Factor (M-CSF), Platelet-derived Growth Factor (PDGF), and phorbol myristate acetate (PMA) enhanced cholesterol efflux from smooth muscle cells by 2-3 times without stimulation of phospholipid efflux. Such stimulation was not observed in macrophages, suggesting that the enhancement of cholesterol efflux involved a cell-specific mechanism. Therefore, we conclude that apolipoprotein-mediated cholesterol efflux is cell line specific, trypsin-sensitive, and cellular regulated process. Further studies need to be done to elucidate unknown mechanisms; i.e., a more reliable method should be employed to quantify the cholesterol content in the plasma membrane which may directly affect the efflux by supplying available cholesterol by various mechanisms; the interaction of apolipoproteins with cells should be examined as well to reveal the function of the surface protein in cholesterol efflux.

REFERENCES

Avira, M., Bierman, E. L. and Oram, J. F. High density lipoprotein stimulates sterol translocation between intracellular and plasma membrane pools in human monocyte-derived macrophages. J. Lipid Res. 1989; 30: 65-76

Badman, J. J., Fuster, V. and Badman, L. Function of high density lipoprotein in the regression of atherosclerosis. Circulation. Supplement III. 1992; 86: 86-94

Baird, A. and Bohlen, P. in Peptide Growth Factors and their Receptors I (eds Sporn. M. B. and Roberts, A. B.) 369-418 (Springer, Berlin, 1990)

Barter, P. J. and Jones, M. E. Kinetic studies of the transfer of esterified cholesterol between human plasma low and high density lipoproteins. J. Lipid Res. 1980; 21: 238-249

Breslow, J. L. Transgenic mouse model of lipoprotein metabolism and atherosclerosis. Proc. Natl. Acad. Sci. USA 1993; 90: 8314-8318

Brown, M. S. and Goldstein J. L. Receptor mediated pathway for cholesterol homeostasis. Science. 1986; 232: 34-47

Bruckdorfer, K. R. and Graham, J. M. Biologic membranes. 1976; (Chapman, D. and Wallach, D. F. H., et al.) pp, 103-152

Castro, G. R. and Fielding C. J. Early incorporation of cell-derived cholesterol into pro-b-migrating high density lipoprotein. Biochemistry. 1988; 27: 25-29

Davis, R. A. J. in Biochemistry of lipids, lipoproteins and membranes. Vance, D. E. and Vance (eds). 1991; p 403-451

Davis, R. A., Dluz, S. M., Leighton, J. K. and Brengaze, V. A. Increased translatable mRNA and decreased lipogenesis are responsible for the augmented secretion of lipid-deficient apolipoprotein E by hepatocytes from fasted rats. J. Biol. Chem. 1989; 264: 8970-8977

Fogelman, A. M., Seager, J. and Haberland, M. E. et al. Lymphocyte-conditioned medium protects human monocyte-macrophages from cholesterol ester accumulation. Proc. Natl. Acad. Sci. U.S.A. 1982; 79: 922-926

Fogelman, A. M., Van Lenten, B. J., Warden, C., and Haberland, M. E. Macrophage lipoprotein receptors. J. Cell Sci. Suppl. 1988; 9: 135-149

Forte, T. M., Goldstein, R. G., Nordhause, R. W. and McCall, M. R. et al. Apolipoprotein AI-cell membrane interaction: extracellular assembly of heterogeneous nascent HDL particles. J. Lipid Res. 1993; 34: 314-324

Garfinkel, A. S. and Schotz, M. C. Lipoprotein lipase. 1987; pp. 335-358, Elsevier, Amsterdam

Glomset, J. in Blood Lipids and Lipoproteins. 1972. (ed. Nelsen, G.) pp 745-787, Wiley, New York

Gold, J. C. and Phillips, M. C. Effect of membrane lipid composition on the kinetics of cholesterol exchange between lipoprotein and different species of red blood cells. Biochem. Biophys. Acta. 1990; 1027: 85-92

Goldstein, J. L., Ho, Y. K. and Basu, S. K., et al. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc. Nati. Acad. Sci. U.S.A. 1979; 76: 333-337

Gordon, T., Castelli, W. P. and Hjortland, M. C., et al. High density lipoprotein as a protective factor against coronary heart disease. Amer. J. Med. 1977; 62: 707-714

Graham, D. L. and Oram, J. F. Identification and characterization of a high density lipoprotein-binding protein in cell membranes by ligand blotting. J. Biol. Chem. 1987; 262: 7439-7442

Hamilton, R. L., William, M. C., Fielding, C. J. and Havel, R. J. Discoidal bilayer structure of nascent high density lipoproteins from perfused rat liver. J. Clin. Invest. 1976; 58: 667-680

Hara, H. and Yokoyama, S. Interaction of free apolipoprotein with macrophages. J. Biol. Chem. 1991; 266: 3080-3086

Henriksen, T., Mahoney, E. M. and Stienberg, D. Enhanced macrophage degradation of biologically modified low density lipoprotein. Arteriosclerosis. 1983; 3: 149-159

Ihm, J., Quinn, D. M., and Busch, S. J., et al. Kinetics of plasma protein-catalyzed exchange of phosphatidylcholine and cholesterol ester between plasma lipoproteins. J. Lipid Res. 1982; 23: 1328-1341

Inaba, T., Gotoda, T., Shimano, H., and Shimada, M., et al. Platelet-derived growth factor induces c-fms and scavenger receptor genes in vascular muscle cells. J. Biol. Chem. 1992; 267: 13107-13112

Jackson, R. L., Morrisett, J. D., Gotto, A. M. Lipoproteins and lipid transport: structural and functional concepts, in Levy R. I. Rifkind BM (eds): Hyperlipidemia, Diagnosis and Therapy. New York, Grune and Stratton, 1977, p 1

Jonas, A. Lecithin:cholesterol acyltransferse. In: Plasma lipoproteins (Gotto, A. M., ED) pp 299-333, Elsevier, Amsterdam.

Jonas, A. Plasma lipoprotein 1987; pp. 299-333

Kodama, T., Freeman, M. and Rohrer, L. et al. Type I macrophage scavenger receptor contains a-helical and collagen-like coiled coils. Nature. 1990; 343: 531-535

Koo, C., Wernette-Hammond, M. E., and Innerarity, T. L. Uptake of canine b-very low density lipoprotein by mouse peritoneal macrophages is mediated by a low density lipoprotein receptor. J. Biol. Chem. 1986; 261: 11194-11201

Mahberg, F. H. and Rothblat, G. H. Cellular cholesterol efflux. J. Biol. Chem. 1992; 267: 4541-4550

McKnight, G. L. and Oram, J. F. Cloning and expression of a cellular high density lipoprotein-binding protein that is up-regulated by cholesterol loading of cells. J. Biol. Chem. 1992; 267: 12131-12141

Mendez, A. J., Oram, J. F. and Bierman, E. L. Protein kinase C as a mediator of high density lipoprotein receptor-dependent efflux of intracellular cholesterol. J. Biol. Chem. 1991; 266: 10104-10111

Miida, T., Fielding, C. J. and Fielding, P. E. Mechanism of transfer of LDL-derived free cholesterol to HDL subfraction in human plasma. Biochemistry. 1990; 29: 10469-10474

Norum, R. A., Lakier, J., Goldstein, S. and Angel, A. et al. Familial deficiency of apolipoproteins A-I and C-III and premature coronary heart disease. N. Engl. J. Med. 1982; 306: 1513-1519

Oram, J. F., Mendez, A. J. and Bierman, E. L. HDL receptor function in cholesterol transport, Carlson LA (ed): Disorders of HDL metabolism. London, Smith-Gordon, 1990, pp 99-104

Oram, J. F., Mendez, A. J., Slotte, J. P. and Johnson, T. F. High density lipoprotein apolipoprotein mediated removal of sterol from intracellular pool but not from plasma membrane of cholesterol-loaded fibroblasts. Arterioscl. Thromb. 1991; 11: 403-414

Ordovas, J. M., Cassidy, O. K., Civeira, F. and Bisgaier, C. L. et al. Familial apoprotein A-I, C-III and C-IV deficiency and premature atherosclerosis due to a deletion of a gene complex on chromosome. J. Biol. Chem. 1989; 264: 16339-16342

Parthasarathy, S., Printz, D. J. and Boya, D. et al. Macrophage oxidation of low density lipoprotein generates a modified form recognized by the scavenger receptor. Arteriosclerosis. 1986; 6: 505-510

Ross, R. The pathogenesis of atherosclerosis: a perspective for the 1990's. Nature. 1993; 362: 801-809

Rothblat, G. and Philips, M. C. Mechanism of cholesterol efflux from cells. J. Biol. Chem. 1982; 257: 4775-4778

Rothblat, G. H., Mahlberg, F. H., Johnson, W. J., and phillips, M. C. Apolipoproteins, membrane cholesterol domains, and the regulation of cholesterol efflux. J. lipid Res. 1992; 33: 1091-1097

Segrest, J. P., Jackson, R. L., Morrisett, J. D. and Gotto, A. M. A molecular theory of lipid-protein interactions in the plasma. FEBS Lett. 1974; 38: 247-258

Sjolund, M., Hedin, U., Sejersen, T., Hedin, C. H. and Thberg, J. Arterial smooth muscle cells express Platelet-derived Growth Factor (PDGF) A chain mRNA, secrete a PDGF-like mitogen, and bind exogenous PDGF in a phenotype-and growth state-dependent manner. J. Cell Biol. 1988; 106: 403-413

Tall, A. R. Plasma lipid transfer protein. J. Lipid Res.1986; 27: 361-367

Thrift, R. N., Forte, T. M., Cahoon, B. E. and Shore, V. G. Characterization of lipoproteins produced by the human liver cell line, Hep G2, under defined conditions. J. Lipid Res. 1986; 27: 236-250

Table I.1. Human plasma apolipoproteins

Apo	KD	plasma mg/dl	Origin	Function
A-I	28	90-130	intestine, liver	LCAT activator
A-II	17	30-50	intestine, liver	?
B-100	549	80-100	liver	receptor binding
B-48	264	<5	intestine, liver	
C-I	6.5	4-7	liver	LCAT activator
C-II	8.8	3-8	liver	LPL, activator
C-III	8.7	8-15	liver	?
E	35	3-6	liver	receptor binding

Chapter II.

Resistance of Smooth Muscle Cells to Assembly of High Density Lipoproteins with Extracellular Free Apolipoprotein and to Reduction of Intracellularly Accumulated Cholesterol

A version of this chapter has been prblished: Akira Komaba, Qianqian Li, Hitoshi Hara, and Shinji Yokoyama. Resistance of Smooth Muscle Cells to Assembly of High Density Lipoproteins with Extracellular Free Apolipoprotein and to Reduction of Intracellularly Accumulated Cholesterol. J Biol. Chem. 267 (1992): 17560-17566

INTRODUCTION

Macrophages and vascular smooth muscle cells can be transformed into foam cells by loading a large amount of cholesterol. The foam cells are the major cell component of atherosclerotic lesions. Cholesterol accumulated in the foam cells cannot be catabolized. Reverse cholesterol transport is the only pathway that can remove the accumulated cholesterol from these cells and transfer it to the liver. In the liver, cholesterol can be catabolized to bile acid. High density lipoproteins (HDL) are considered to be the most potent factor to prevent atherosclerosis (Stein, et al. 1976; Ho, et al. 1980). The low level of circulating HDL cholesterol is associated with the higher risk of coronary diseases in epidemiological studies (Gordon, et al. 1990). The mechanism of the "reverse cholesterol transport" remains unclear. It is observed that HDL-mediated cholesterol efflux is a rapid bi-directional movement. When HDL is incubated with cholesterol-loaded membranes, the cholesterol flux between HDL and the membrane reaches balance very fast and the kinetic profile is consistent with the diffusion movement (Phillips et al. 1980; Rothblat, et al. 1982). Thus, HDL-mediated cholesterol efflux is mostly mediated through a non-specific exchange mechanism. Net removal of cholesterol from cells by HDL only occurs in cholesterol enriched cell membranes. If HDL generally induces a net cellular lipid efflux as assumed, a more specific explanation may have to be introduced for the lipid efflux beyond the nonspecific mechanism mentioned above.

Yokoyama's group has recently demonstrated that free apolipoproteins with multi-segments of amphiphilic a-helix, mediate net cholesterol efflux by generating HDL-like particles and reduce cellular cholesterol ester (Hara and Yokoyama , 1991). The Km of the interaction between cell membranes and apolipoproteins is low. The structure requirement of apolipoproteins for this reaction is the presence of more than four amphiphilic helical amino acids (Hara and Yokoyama, 1991; 1992). Therefore, a wide variety of apolipoproteins with similar physicochemical properties, such as human apolipoprotein A-I, A-II, A-IV, E, and insect apoLpIII, are capable of carrying out such an interaction. These apolipoproteins are exchangeable among lipoproteins. It allows them to associate with and dissociate from the lipoprotein surface and interact with the cell membrane, which eventually contributes to the overall cellular lipid removal by lipoprotein (HDL). Because these apolipoproteins mediate a low Km reaction and act as an active exchangeable form among lipoprotein surfaces, it is assumed that under physiological conditions apolipoproteins can partially dissociate from the HDL surface and participate in reverse cholesterol transport. In this way, the apolipoprotein-mediated lipid efflux may have physiological relevance by functioning in the prevention of atherosclerosis and the formation of new HDL-like particle.

In order to establish the physiological relevance and mechanism of the reaction between apolipoproteins and cells, we have studied other types of cells rather than macrophage-derived foam cells, such as aortic smooth muscle cells (rat, monkey and rabbit), and human skin fibroblasts. As demonstrated by previous studies, those cells all play roles in atheromatous vascular lesions in different stages (Gerrity, et al. 1981; Ross 1986; Johnson, et al. 1988; and Castro, et al. 1992).

MATERIALS AND METHODS

1. Lipoprotein

HDL was isolated from fresh human plasma as densities 1.063-1.21 g/ml in NaBr through sequential ultracentrifugation. The HDL preparation was dialyzed against 0.1 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl, and stored at 4 °C under argon. The purity was confirmed through the criteria of electrophoretic mobility in a 0.5% agarose gel. The lipid composition was measured by using enzymatic assay kits (Wako Pure Chemicals, Osaka). Apolipoprotein composition was analyzed by polyacrylamide gel electrophoresis (for details see chapter 3).

2. Apolipoproteins

Apolipoproteins were obtained from fresh human plasma. VLDL and LDL were removed by ultracentrifugation in density 1.063 g/ml KBr at 50,000 rpm for 24 h. HDL was obtained as density about 1.12-1.20 g/ml in KBr at 50,000 rpm for 48 h and then HDL solution was dialyzed against 1 mM EDTA, pH 7.4. HDL was delipidated with ethanol/ether (2/3, v/v) three times at -20 °C.

The delipidated sample was dissolved in 30 mM Tris-HCl pH 8.0, 6 M urea and applied to DEAE-cellulose ion-exchange chromatography (Tajima, et al. 1983). The fractions containing the respective pure apolipoproteins (as checked by SDS-gel) were pooled and dialyzed against PBS, dried down and stored in -70 °C as a powder under argon. All the apolipoprotein solutions used in experiment were not kept at 4 °C longer than 3 weeks.

3. Preparation and Modification of [^{3}H]-cholesteryl oleate-labeled LDL

Fresh human blood was mixed with ethylenediaminetetraacetic acid (1%). Plasma was obtained after centrifugation, 3000 rpm for 25 min at 4 °C. The plasma was then centrifuged at 10^6 x g for 16 h at 4 °C, and the bottom fraction (d=1.006 g/ml) was recovered. Phosphatidylcholine (from Sigma) 6 mg and [$^{1,2-3}$H]-cholesteryl oleate (from Amersham Canada) 8.58 mg (600 μCi) were mixed and then dried down. A 20 ml of sodium phosphate buffer (containing 0.15 M NaCl, pH 7.4) was added and then sonicated for 30 min on ice bath under a nitrogen stream. The bottom fraction (d=1.006 g/ml) of plasma was mixed with the above sonicated lipid solution. Dithionitrobenzoic acid (DTNB), aprotinin, and gentamycin were added to give final concentration of 2 mM, 20 units/ml, and 0.1 mg/ml, respectively. The mixture was incubated at 37 °C for 24 h. The incubation mixture was applied to a dextran sulfate-cellulose column which had been equilibrated with the same sonication buffer. The column was washed with the buffer and then the bound fraction was eluted with 50 ml of the same buffer containing an NaCl concentrations gradient from 0.15 to 0.5 M. Alternatively, the bound fraction was eluted with 0.35 M NaCl solution in the same buffer. Each 2 ml fraction was collected and analyzed for radioactivity and cholesterol. The peak fractions of cholesterol eluted with high NaCl concentration were combined and density were adjusted to 1.063 g/ml. The solution was then centrifuged at 10^6 x g for 20 h at 4 °C. The top fraction was collected, dialyzed against the buffer containing 0.15 M NaCl, and analyzed for cholesterol, phospholipid, triglyceride and protein. The electrophoretic mobility of lipoprotein in the top fraction was examined in 5% polyacrylamide gel.

The labeled LDL was acetylated by the method described by Basu et al. (Basu, et al. 1976). In a typical preparation, 1 ml of 0.15 M NaCl containing 16 mg of LDL protein was added to 1 ml of a saturated solution of sodium acetate with continuous stirring in an ice bath. Next, acetic anhydride was added in multiple small aliquots over a period of time with continuous stirring. After the addition of a total mass of acetic anhydride (equal to 1.5 times the mass of protein used), the mixture was stirred for an additional 30 min without further additions. The reaction solution was then dialyzed for 24 h at 4 °C against 12 liters of buffer containing 0.15 M NaCl and 0.3 mM EDTA, pH, 7.4.

LDL was cationized as described by the same author (Basu, et al. 1976). An aliquot of 16 mg LDL protein was added to 1 ml of 2 M N,N-dimethyl-1,3-propanediamine adjusted to pH 6.5 with HCl. Next, 100 mg 1-ethyl-3(3-dimethylaminopropyl) carbodiimide HCl was added, and the pH was maintained at 6.5 by addition of 0.2 M HCL with continuous stirring until the reaction had completed, as indicated by no measurable change in pH. The reaction mixture was left overnight at 4 °C and then dialyzed for 24 h at 4 °C against 12 liter of buffer containing 0.1 M NaCl and 0.3 mM of EDTA, pH 7.4. The final specific radioactivity is about 10,000 dpm/µg protein. The chemical modifications of LDL were confirmed by electrophoretic mobility in agarose gel.

4. Loading cells with cholesterol

4.1. Macrophages

Mouse peritoneal macrophages were obtained from peritoneal lavage as described (Hara and Yokoyama, 1991). The cells were washed with cold PBS and then the cells were incubated with 20% Fetal Calf Serum (FBS) at 37 °C for 2 h to allow cells to grow on the well. The monolayer was washed and loaded with radiolabeled cholesterol according to method described by Hara by incubating the cells with the acetylated LDL labeled at cholesterol ester (50 µg/ml) in 1 ml RPMI 1640 medium (Flow Laboratories) containing 2 mg of bovine serum albumin (BSA) for 24 h (Hara and Yokoyama, 1991). After loading, the cells were washed 3 times with medium and reincubated without lipoprotein for another 24 h for cholesterol incorporation.

4.2. Smooth muscle cells

Smooth muscle cells were obtained from the entire thoracic aorta from a Spragure-Dawley rat and a New Zealand White rabbit (2-week old). After mechanically stripping the adventitia as completely as possible. The remaining tissue was incubated in 15 ml of culture medium containing type I collagenase 40 mg (obtained from Sigma), and bovine serum albumin 15 mg, at 37 °C for 120 min. The aorta was cut and opened, removing the intima by scratching with a metal scraper and cutting into small pieces. The tissue pieces were incubated in the same collagenase medium at 37 °C for about 120 min until the cells were detached. The cell mixture was centrifuged at 1000xg for 5 min at 4 °C, and the pellet was washed twice with the medium without collagenase. The cells were incubated in a large plastic culture dish (10 cm) with RPMI 1640 medium containing 10% FBS (Flow Laboratories) until cells became subconfluent. The cells were harvested with 0.125% trypsin solution, washed, and transferred into three 10-cm dishes. After 6-9 passages with the same procedure, the cells were seeded in small culture dishes (3.5-cm) as 1-2x10^4 cells/dish. Aortic smooth muscle cells were also prepared from a cynomologus monkey (Maraca fascicularis) by Dr. H. Kasuya, Department of Neurosurgery and generously provided by Dr. B. Vollrath, Department of Pharmacology, University of Alberta. Cells were maintained in 10% fetal calf serum medium until they became subconfluent and then incubated in the medium containing 10% lipoprotein-deficient calf serum for 24 h. The cells were then incubated with cationized LDL containing radiolabeled cholesterol ester. Rat smooth muscle cells were incubated for 1 week for cholesterol loading, and monkey and rabbit cells were incubated for 2 days. The labeled cells were washed three time with medium and incubated in the minimum essential medium with 0.2% albumin for another 24 h.

4.3. Human fibroblasts

Detroit 551 normal human fibroblasts were seeded in a culture dish (3.5-cm) as 1-2x10^4 cells/dish and incubated in 10% fetal calf serum medium for 4-5 days until they became subconfluent. The cells were first incubated in medium containing 10% lipoprotein-deficient calf serum for 24 h, and then incubated with LDL containing the labeled cholesterol ester for 24 h, washed, and maintained in the medium with 0.2% BSA for 24 h.

5. Incubation of Cholesterol loaded cells with ApoA-I, A-II and HDL

The cholesterol loaded cells prepared as above were washed and then incubated with 1 ml of 0.2% BSA RPMI 1640 medium containing apoA-I, A-II and HDL. Before being applied to the cell cultures, apoA-I and A-II were preincubated in 37 °C for 1 h. After 24 h incubation unless otherwise specified, the culture medium was collected and the cellular remnants were removed by centrifugation at 3000xg for 2 min. The lipids were extracted from the medium as described (Bligh and Dyer 1959). Cellular lipids were also extracted with Hexane/Isopropyl alcohol (3:2, v/v) (Hara and Yokoyama, 1991). Aliquots of lipids were analyzed for total radioactivity. The radioactivity in unesterified and esterified cholesterol were counted after the lipids were separated by thin layer chromatography. Another aliquot of cellular lipid from control samples was dried and completely hydrolyzed by incubating in 50 μl of 0.05 N KOH at 37 °C for 2 h. Total cholesterol was assayed with an enzymatic assay kit (Wako Pure Chemicals, Osaka) after neutralizing the solution with 50 μl of 0.05N HCl. Specific radioactivity in cellular cholesterol calculated from results above was used for determination mass of unesterified and esterified cholesterol in the cell. Cellular protein was determined by the method of Lowry et al. (Lowry, et al. 1951). The variations of the data for experimental points were within 10% when performed in duplicate. Each data point shown in the figures therefore represents a single experimental point.

6. Density gradient ultracentrifugation

After incubation with apolipoproteins or HDL, particles formed in the culture medium were analyzed by sucrose density gradient ultra centrifugation in a Beckman TL 100 Table Top Ultracentrifuge with TLA 100.3 rotor at 200,000xg for 16 h (Hara and Yokoyama, 1991; 1992), for details see next chapter.

RESULTS

1. Cholesterol efflux from cholesterol loaded macrophages and fibroblasts

The previous experiments showed that apoA-I and A-II both induced net cholesterol efflux from macrophages with similar profile. HDL as a reference for apoA-I and A-II also induced apparent release of cellular cholesterol (Hara and Yokoyama, 1991). For apoA-I and A-II, Vmax were calculated as the average of the maximum rate of efflux, and Km was graphically obtained as the apolipoprotein concentration at half of the Vmax. The Vmax rate for apoA-I and A-II were both about 25% of that of HDL, which may be equivalent to the net efflux of cellular cholesterol from cholesterol-loaded cells in this reaction (Johnson, et al. 1988). While the Km for HDL was greater than 30 µg/ml protein, the Km for apoA-I and A-II was only 7 and 4 µg/ml, respectively. After the cells had been incubated with apoA-I the medium was collected and analyzed by density gradient ultracentrifugation. The results showed that cholesterol was present in the lipoprotein fraction with a density of 1.13 g/ml, which was similar to the density of HDL.

Cholesterol efflux from cholesterol loaded human fibroblasts mediated by apoA-I, A-II and HDL was essentially the same as that from macrophages. The Km values for apoA-I and A-II were 1µg/ml. The Vmax of apoA-I and A-II were 25% of Vmax for HDL. Concomitant with the release of cholesterol into medium, cellular cholesterol was decreased after the cells were exposed to free apolipoproteins. The decrease of cellular cholesterol in apoA-I and A-II mediated efflux was predominantly esterified cholesterol. The majority of efflux cholesterol was in the fraction with the density 1.1-1.15 g/ml in the medium, demonstrating that HDL-like particles were generated through the interaction of free apolipoprotein with cholesterol loaded fibroblasts.

2. Cholesterol efflux from smooth muscle cells

Figure II.1 shows cholesterol efflux from monkey and rabbit aortic smooth muscle cells. The rate of cholesterol efflux mediated by apoA-I and A-II was very low. The saturating points for cholesterol efflux were only about 1% of total cholesterol. However the Km values were the same as that of fibroblasts. HDL induced an apparent cellular cholesterol efflux with approximately the same Km as that for other cells. Therefore, the rate of efflux with apoA-I and A-II was less than 10% of that with HDL and lower than one-third of that of macrophages and fibroblasts when compared upon the basis of the relative rate of Vmax for apoAI and A-II to the

Vmax for HDL. As a result, the decrease of cellular cholesterol content by free apolipoproteins was not significantly demonstrated. The decrease of cellular cholesterol by HDL in smooth muscle cell was in the same manner as that of fibroblasts. The density gradient profile showed that HDL-like particles were generated by apoA-I and A-II with the same density as that in macrophages and fibroblasts.

3. Analysis of the parameters of cholesterol efflux from various types of cells.

Table II.1 summarizes the results of analysis of the Vmax values from the various cells. The Vmax values were standardized by intracellular unesterified cholesterol content in each experiment as an apparent first-order rate constant because Johnson et al suggested that a first-order rate constant of cellular cholesterol efflux to HDL and free apoA-I and A-II is independent of cellular unesterified cholesterol levels (Johnson et al. 1988). The results show Vmax values for free apolipoproteins seem to be cell-line specific, while the parameter was more or less similar for HDL among these three types of cell. The Vmax for apoA-I was 3.4-6.8%, 1.4-1.5% and 1.8%, and for apoA-II was 2.2-7.5%, 0.4% and 2.5% of cellular cholesterol for rat, monkey and rabbit smooth muscle cells respectively. On the other hand, the Vmax of apoA-I for fibroblasts and macrophages were about 42% to 60%. However, the value for HDL was 60-90% cellular cholesterol with smooth muscle cells, 139% with fibroblasts, and 140-180% with macrophages. Accordingly, Vmax values of apoA-I and A-II relative to that of HDL showed cell specificity. These values were lower than 10% in smooth muscle cells, but greater than 30% in fibroblasts and macrophages. Thus, smooth muscle cells are resistant to net cholesterol release induced by apolipoproteins, while cholesterol efflux mediated by HDL does not much differ among the three cell types examined.

Table II.2 summarizes the apparent Km values for cholesterol efflux from cells induced by apoA-I, A-II and HDL. For HDL there was no significant difference among the three types of cell. Since Vmax is similar in each type (Table I), the nature of interaction of HDL with cells seems consistent throughout these cells in terms of cellular cholesterol exchange. However, both apoA-I and A-II had very small Km for smooth muscle cells and fibroblasts in comparison with the Km for macrophages.

Table II.3 is the overall summary of the data for interaction of apoA-I and A-II with smooth muscle cells, fibroblasts, and macrophages, using the apparent efflux rate of cellular cholesterol to HDL as a reference. The interaction of HDL with each cell type was essentially the same as characterized by the apparent Vmax and Km values. The Vmax of cholesterol efflux from smooth muscle cells is about 10 times lower than that of fibroblasts and macrophages after the rate was standardized for cellular unesterified cholesterol pool. Consequently, the rate of cholesterol efflux is also significantly lower from smooth muscle cells when standardized for the apparent Vmax values for HDL. The Km value was, however, high for macrophages and very low for smooth muscle cells and fibroblasts: the Km for macrophages and smooth muscle cells were 1/500 and 1/1500 of plasma apoA-I and A-II concentration, respectively.

For summary, free apolipoproteins interact with each of these three types of cell, generating new HDL-like particles and inducing cellular cholesterol efflux. However, smooth muscle cells seem resistant to this reaction as indicated. Both fibroblasts and macrophages exhibited high Vmax of net cholesterol efflux which may be almost equivalent to the net efflux from cholesterol-loaded cells to HDL. However, the Km for macrophages was significantly higher than that for smooth muscle cells and fibroblasts. Therefore, the results suggest that smooth muscle cells are resistant to cellular cholesterol removal by extracellular free apolipoproteins.

DISCUSSION

In order to determine from which type of atherosclerotic plaques cell the accumulated cholesterol is preferably removed, we have studied a generation of new HDL-like particles through the interaction of apolipoprotein with smooth muscle cells (rat, monkey and rabbit), human skin fibroblasts and mouse peritoneal macrophages. The efflux of prelabeled cellular cholesterol to HDL was also measured for reference. Extracellular apoA-I and A-II interacted with all three cell types and generated HDL-like particles with cellular lipids. The intracellular cholesterol was visibly reduced when the lipid removal was sizable. The Vmax of cholesterol efflux to HDL and free apolipoproteins were standardized for cellular unesterified cholesterol pool according to the finding by Johnson et al (Johnson, 1991) that the first-order rate constant for cellular cholesterol efflux from fibroblasts to HDL was independent of the cellular unesterified cholesterol content. In our experiment, the standardized rate was indeed independent of cellular unesterified cholesterol content, not only for the efflux to HDL but also for the rate of new generation of HDL-like particles by free apolipoproteins. These standardized rate of cholesterol efflux to HDL varied only by less than 2-fold among the three types of cell, in order from low to high: smooth muscle cells, fibroblasts and macrophages. However, the standardized rates for generation of HDL-like particles by free apolipoproteins with smooth muscle cells was as low as one-tenth of that with fibroblasts and macrophages. In contrast, the affinity of free apoA-I and A-II represented by Km of the reaction was much higher for smooth muscle cells and fibroblasts than that for macrophages. The Km for macrophages was as low as 1/500 of the plasma apoA-I concentration but for smooth muscle cells and fibroblasts were even lower (1/1500 of plasma concentration) (Hara and Yokoyama, 1991). On the other hand, the apparent Km values of HDL for cellular cholesterol efflux were similar for the three types of cell.

The results probably indicate that the apparent efflux of cholesterol to HDL is mediated through a nonspecific mechanism such as diffusion of cholesterol through the aqueous phase as suggested by Phillips, Rothblat and his coworkers (Johnson et al. 1991). The efflux from this pathway is bi-directional exchange. The net cholesterol efflux from this pathway was only one-third of the total cholesterol efflux and only occurred in cholesterol loaded fibroblasts (Johnson, et al. 1988). Therefore, no matter how much of cellular cholesterol efflux was observed in HDL-mediated lipid efflux, it did not necessarily mean that there was significant net removal of cellular cholesterol by HDL. The results also demonstrated that such a cholesterol exchange between the cells and lipoproteins was essentially the same among the cells tested. On the other hand, cholesterol efflux mediated by extracellular free apolipoproteins through the generation of HDL-like particles was a pure "net" efflux. The Kd of apoA-I and A-II for interaction with the surface of phosphatidylcholine/triolein microemulsion was the same order as the Km for macrophages and higher than that for smooth muscle cells and fibroblasts (Hara and Yokoyama, 1991;

Tajima and Yokoyama et al., 1983) Therefore, if the Km value of apolipoprotein for this reaction indeed indicated the affinity of these apolipoproteins for the cell surface, the small amount of apolipoproteins dissociated from HDL surface may be enough to interact with the cell surface and mediate cholesterol efflux. Thus, this mechanism may explain the preferable net cholesterol efflux to HDL. Since the maximum rate of cholesterol efflux induced by free apolipoproteins was approximately one-third of the apparent efflux rate to HDL with macrophages and fibroblasts, the net efflux mediated by HDL may be indeed consistent with the new HDL generation mediated by apolipoproteins. This may also explain why the rate constant for cellular cholesterol efflux from smooth muscle cells to HDL was relatively lower because the rate of new HDL generation was extremely low. Thus, from the results of these experiments, it was demonstrated that smooth muscle cells were very resistant to the net removal of cellular cholesterol by the mechanism of generating new HDL through free apolipoproteins.

The HDL generated by the interaction of free apolipoproteins with cellular lipids of macrophages had previously shown preb electrophoretic mobility (Hara and Yokoyama, 1991). This reaction had also been shown to mediate the transfer of cellular cholesterol to artificial lipoproteins from macrophages (Hara and Yokoyama, 1992). Fielding and his colleague demonstrated that cellular cholesterol first occurred in a preβ-HDL fraction when cholesterol loaded fibroblasts were incubated with human plasma (Castro and Fielding, et al. 1988). Our results suggested that the new HDL-like particles generated by apolipoproteins may be involved in such an observation.

The mechanism for the significant difference of apparent Vmax and Km of generation of HDL-like particles among various cell types is unknown. The membrane lipid composition may affect the lipid-apolipoprotein interaction. The membrane surface proteins may also play a role in lipid efflux as a modulator. Other physicochemical parameters such as flexibility or fluidity of the membrane could affect the now HDL-like particles as well.

REFERENCES

Basu, S. K., Goldstein, J. L., Anderson, R. G. W., and Brown, M. S. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. Proc. Natl. Acad. Sci. U. S. A. 1976; 73: 3178-3182

Bligh, O. H. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911-917

Castro, G. R., and Fielding, C. J. Early incorporation of cell-derived cholesterol into pre-b-migrating high density lipoprotein. Biochemistry. 1988; 27: 25-29

Fielding, C. J., and Fielding, P. E. Evidence for a lipoprotein carrier in human catalyzing sterol efflux from culture fibroblasts and its relationship to lecithin:cholesterol acyltransferase. Proc. Natl. Acad. Sci. U. S. A. 1981; 78: 3911-3914

Francis, G. A., Ko, K. W. S., Hara, H., and Yokoyama, S. Regulation of the uptake of high density lipoprotein-originated cholesterol ester by $HepG_2$ cells: role of low density lipoprotein and plasma lipid transfer protein. Biochim. Biophys. Acta 1991; 1084: 159-166

Funahashi, T., Yokoyama, S. and Tamamoto, A. Association of apolipoprotein E with the low density lipoprotein receptor: demonstration of its co-operativity on lipid microemulsion particle. J. Biochem (Tokyo). 1988; 105: 582-587

Gerrity, R. G. The role of the monocyte in atherogenesis. Am. J. Pathol. 1981; 103: 181-190

Gerrity, R. G. The pathogenesis of atherosclerosis - an update. New Engl. J. Med. 1986; 314: 488-500

Gerrity, R. G. The role of the monocyte in atherogenesis. Am. J. Pathol. 1981; 103: 191-200

Gordon, D. J. (1990) Epidem. and Metab. Clinic N. America 19, 299-309

Hara, H., and Yokoyama, S. Interaction of free apolipoproteins with macrophages. J. Biol. Chem. 1991; 266: 3080-3086

Hara, H., and Yokoyama, S. Role of apolipoproteins in cholesterol efflux from macrophages to lipid microemulsion: proposal of a putative model for the pre-b- high density lipoprotein pathway. Biochemistry. 1992; 31: 2040-2046

Ho, Y. K., Brown, M. S., and Goldstein, J. L. Hydrolysis and excretion of cytoplasma cholesterol by macrophages: stimulation by high density lipoprotein and other agents. J. Lipid Res. 1980; 21: 391-398

Johnson, W. I., Mahlberg, F. H., Rothblat, G. H. and Philips, M. S. Cholesterol transport between cells and high density lipoprotein. Biochem. Biophys. Acta. 1991; 1085: 273-298

Johnson, W. J., Mahlberg, F. H., Chacko, G. H., Phillips, M. C., and Rothblat, G. H. The influence of cellular and lipoprotein cholesterol contents on the flux of cholesterol between fibroblasts and high density lipoprotein. J. Biol. Chem. 1988; 263: 14099-14106

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein measurement with the folin phenol reagent. J. Biol. Chem. 1951; 193: 265-275

Nishikawa, O., and Yokoyama, S. A method for direct incorporation of radiolabeled cholesterol ester into human plasma low density lipoprotein preparation of trace substrate for cholesterol ester transfer reaction between lipoproteins. J. Biochem. (Tokyo). 1985; 99: 295-301

Rothblat, G. H. and Phillips, M. C. Mechanism of cholesterol efflux. J. Biol. Chem. 1982; 257: 4775-4782

Stein, O. V. and Stein Y. Cholesterol and sterol synthesis in human skin fibroblasts and rat aortic smooth muscle cells exposed to lipoprotein-deleted serum and high density lipoprotein mixtures. Biochim. Biophys. Acta. 1976; 431: 347-358

Tajima, S., Yokoyama, S., and Yamamoto, A. Effect of particle size on association of apolipoprotein with lipid. J. Biol Chem. 1983; 258: 10073-10082

Yokoyama, S., Kawai, Y., Tajima, S. and Yamamoto, A. Behavior of human apolipoprotein E in aqueous solutions and at interfaces. J. Biol. Chem. 1985; 260: 16375-16382

Figure. II.1. Cholesterol efflux from monkey (A) and (B) aortic smooth muscle cells. Panel A and panel B corresponds to the experiment with smooth muscle cells monkey 2 and rabbit smooth muscle cells in Table I, respectively. After the cells were labeled by incubating with 15 μg/ml of cationized LDL for 3 days (A) and 2 days (B), cholesterol released from the cells was measured in the presence of apoA-I (○), apoA-II (●), and HDL (Δ).

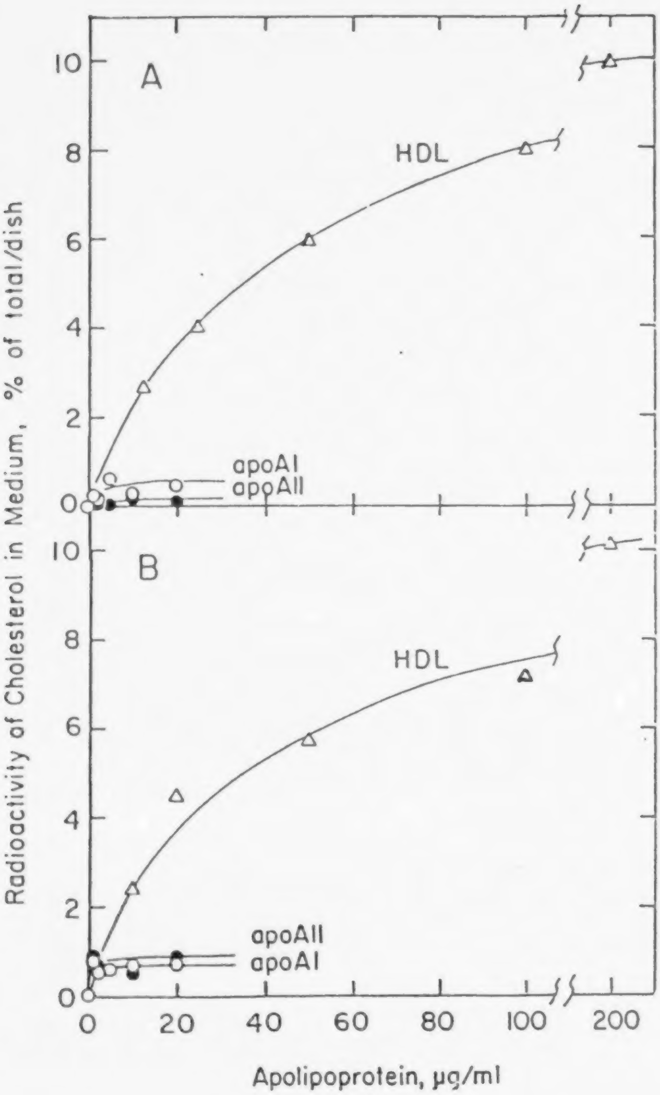
A
B
HDL
apoAI
apoAII
HDL
apoAII
apoAI
Radioactivity of Cholesterol in Medium, % of total/dish
Apolipoprotein, µg/ml
0
2
4
6
8
10
0
20
40
60
80
100
200

Figure II.1

Table II.1. Vmax for HDL was obtained by double reciprocal plot of the data using a least square linear regression. Vmax was obtained for apoA-I and apoA-II as the average of the rates with protein concentrations from 5 to 20 µg/ml. The rates are for 24 h except for experiment 3 with macrophages (5 h). Cholesterol was loaded by incubating cells for 24 h with labeled lipoproteins containing; LDL, 200 µg protein for rat smooth muscle cell 2,3, and fibroblast 1, 2; cationized LDL, 10 and 50 µg protein for rat smooth muscle cell 4 and 5, respectively, and 15 µg for monkey and rabbit smooth muscle cells; acetylated LDL, 50 µg protein for macrophage 1, 2, 3; and phospphatidylcholine/triolein/cholesterol oleate microemulsion (11.3/7.6/6.9, w/w/w) preincubated with human apolipoprotein E (22% of phospholipid by weight), 565 µg phospholipid and 125 µg protein, for rat smooth muscle cells 1. Cellular protein (protein/dish), cellular total cholesterol (t.chol./prot.) and unesterified cholesterol (f.chol/prot.) represent the initial condition for cholesterol efflux experiments. Vmax/f.chol. is the efflux rate standardized for the initial cellular unesterified cholesterol pool in each experiment condition. Vmax/Vmax is the efflux rate relatively to the Vmax of the efflux to HDL.

Table II.1. Apparent Vmax values for three types of cell in apoA-I, apoA-II and HDL mediated cholesterol efflux

experiment			protein/dish (μg)	t.chol./prot. (μg/mg)	f.chol./prot. (μg/mg)	V_{max}/f.chol. (%)			$V_{max}/V_{max(HDL)}$ (%)	
						ApoA-I	ApoA-II	HDL	ApoA-I	ApoA-II
SMC[a]	rat	1	90.0 ± 7.0	15.7 ± 2.3	12.1 ± 0.2	6.0 ± 2.4	7.5 ± 0.4	59	10.2 ± 4.1	12.7 ± 0.7
		2	49.2 ± 4.9	55.5 ± 5.9	36.2 ± 3.9	6.8 ± 1.5	6.0 ± 3.4	86	7.9 ± 0.2	7.0 ± 4.0
		3	98.0 ± 10.4	32.0 ± 3.0	22.4 ± 2.1	6.1 ± 1.7	5.6 ± 3.6	84	7.3 ± 0.2	6.7 ± 4.3
		4	134.4 ± 9.3	75.2 ± 10.4	18.7 ± 2.6	4.6 ± 0.1	3.7 ± 0.3	92	5.0 ± 0.1	4.0 ± 0.3
		5	187.8 ± 10.3	135.7 ± 16.0	18.4 ± 2.5	3.4 ± 0.6	2.2 ± 0.4	59	5.8 ± 1.0	3.7 ± 0.7
	monkey	1	19.4 ± 3.2	--	(52 % of total)	1.5 ± 0.6	0.4 ± 0.1	42	3.7 ± 0.4	0.9 ± 0.2
		2	10.1 ± 0.6	220.0 ± 15.0	54.6 ± 3.6	1.4 ± 0.8	0.4 ± 0.3	47	3.0 ± 1.5	0.8 ± 0.5
	rabbit	1	24.5 ± 7.8	--	(36 % of total)	1.8 ± 0.5	2.5 ± 1.2	31	5.8 ± 1.5	8.0 ± 3.9
fibroblasts		1	71.1 ± 7.2	66.8 ± 0.3	31.9 ± 1.0	42.9 ± 3.1	35.1 ± 1.8	139	30.9 ± 2.2	25.3 ± 1.3
		2	53.9 ± 10.7	81.0	41.0	42.0 ± 3.0	42.4 ± 6.6	139	30.2 ± 2.8	30.5 ± 4.7
macrophages		1	7.2 ± 3.4	209.6	70.6	59.8 ± 9.0	--	189	31.6 ± 4.8	--
		2	11.0 ± 3.3	--	(34% of total)	46.2 ± 6.0	--	143	32.3 ± 4.2	--
		3	77.3 ± 6.4	303.2 ± 4.4	71.6 ± 2.3	10.3/5h	18.4/5h	46/5h	22.4	44.0

[a] smooth muscle cells

Table II.2. Apparent Km values of radioactive cholesterol efflux from various types of cell

cell type		apoA-I	apoA-II	HDL
SMC[a]	rat	1[b]	1[b]	65 ± 21[c]
	monkey	<1.25	<1.25	53, 50
	rabbit	<1.25	<1.25	36
fibroblast		1[d]	1[d]	46[d]
macrophage		6.4 ± 2.8[e]	3.2 ± 0.9[f]	40[g]

[a] smooth muscle cell

[b] Average of experiments 3 and 4 because other experiments did not cover the range of K_m.

[c] Mean ± S.D. of the data of experiments 1 - 5.

[d] Average of experiments 1 and 2 because other experiments did not cover the range of K_m.

[e] Calculated from the data of experiments 1 - 3 (mean ± S.D.).

[f] Taken from references 17-19.

[g] Average of experiments 1 and 3 because experiment 2 does not cover the range of K_m.

Table II.3. Summary of parameters for cellular cholesterol efflux from the three types of cells induced by apoA-I, apoA-II and HDL

cell type		HDL		apoA-I			apoA-II		
		V_{max}/f.chol (%)	K_m (μg/ml)	V_{max}/f.chol (%)	$V_{max}/V_{max(HDL)}$ (%)	K_m (μg/ml)	V_{max}/f.chol (%)	$V_{max}/V_{max(HDL)}$ (%)	K_m (μg/ml)
SMC[a]	rat	76 ± 16	65 ± 21	5.4 ± 1.4	7.2 ± 2.0	1	5.0 ± 2.1	6.8 ± 3.6	1
	monkey	45	51	1.5	3.3	<1.25	0.4	0.8	<1.25
	rabbit	31	36	1.8	5.8	<1.25	2.5	8.0	<1.25
fibroblast		139	56	42.4 ± 3.4	30.5 ± 2.5	1	39.7 ± 6.4	28.6 ± 4.6	1
macrophage		166	40	53.0 ± 13.5	31.9 ± 8.1	6.4 ± 2.8	45.5 ± 5.5[b]	27.4	3.2 ± 0.9

[a] smooth muscle cell

[b] Data are taken from references 17-19.

Chapter III.

Cholesterol Is Poorly Available for Free Apolipoprotein Mediated Cellular Lipid Efflux from Smooth Muscle Cells

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INTRODUCTION

In the early stage of atherosclerosis (fatty streak stage), macrophages play a major role in accumulation of cholesterol by taking up modified LDL and turn to the appearance of foam cells. Then smooth muscle cells may play a role in advancing to next stage by proliferating in lesions, accumulating lipids and forming foam cells. The cholesterol accumulated in macrophages and smooth muscle cells can not be catabolized, and thus removal of accumulated cholesterol from these cells is important to prevent the development of atherosclerosis. As reported that the accumulation of cholesterol ester in macrophages seems reversible to be removed in some extent when reducing the level of plasma cholesterol (Yamamoto et al., 1989), and exposing the cells to HDL (Burns, et al. 1969; Werb, et al. 1971; Stein, et al. 1976; Ho, et al., 1980). On the other hand, the foam cells derived from smooth muscle cells seem to be relatively resistant to removal of cellular cholesterol by HDL (Tabas, et al. 1984; Savion, et al. 1989). However, the mechanism of cellular cholesterol efflux has not been fully understood. Several mechanisms have been proposed. One of them is the diffusion mechanism (nonspecific mechanism) (Rothblat et al. 1982; Johnson, et al. 1986, 1988; Karlin, et al. 1987). By this mechanism, cholesterol molecules diffuse from cell membrane to lipoproteins down the cholesterol gradient. Therefore, net cholesterol efflux occurs only in cholesterol enriched membranes. The second proposed mechanism is HDL-binding protein mediated cholesterol efflux. By an unknown mechanism, the binding of HDL to cell surface promotes translocation of cholesterol from the intracellular cholesterol pool to the cell membrane pool (Slotte, et al. 1987; Aviram, et al. 1989; McKnight, et al. 1992). Cholesterol on the cell membrane is available for efflux.

We have demonstrated that many apolipoproteins with amphiphilic α-helical segments (apoA-I, A-II, A-IV, E, and apolipophorin III from insect hemolymph) mediate net cholesterol and phospholipid efflux from macrophages, generating HDL-like particles. The Km of this reaction is only 1/500 of plasma apoA-I concentration. Therefore, the proposal is that a small amount of apolipoprotein dissociated from lipoproteins can interact with cell surface to mediate net cholesterol efflux by generating preb-HDL particles and contribute to the overall lipid efflux (Hara, et al. 1992). The particles formed through the reaction possess similar mobility and lipid components to preb-HDL (Hara, et al. 1991; 1992). Cellular cholesterol occurs on the preb-HDL prior to other plasma lipoproteins when fibroblasts are incubated with human plasma (Castro, et al. 1988), suggesting cholesterol efflux from cells to free apolipoproteins may be the first step in the process of cholesterol removal. These particles have been observed with electron microscope when CHO cells are incubated with free apolipoprotein (Forte, et al. 1993). Therefore the reaction has physiological relevance in such terms that the low concentration of lipid-free apolipoprotein present in the intestinal fluid or dissociated from lipoprotein surface may mediate net cholesterol efflux to generate new HDL and contribute to regression and prevention of atherosclerosis.

Interestingly, we have discovered that smooth muscle cells are highly resistant to net cholesterol efflux mediated by lipid-free apolipoprotein, in comparison to macrophages and fibroblasts. However, the rate of cellular cholesterol efflux through the nonspecific exchange pathway is only slightly lower than that of other cells (Komaba, et al. 1992). The affinity of apolipoprotein for smooth muscle cell surface is even higher than that for the macrophages in terms of Km for cholesterol efflux reaction (Chapter 2). This finding may be the evidence for the possible resistance of smooth muscle cells to regression of atherosclerosis. In this chapter, we studied the underlying mechanism of this resistance and the results also showed that smooth muscle cells were as reactive as macrophages to lipid-free apoA-I in terms of cellular phospholipid efflux. However, cellular cholesterol is extremely poorly available for this reaction, resulting in very poor net cholesterol efflux through this mechanism.

MATERIALS AND METHODS

1. Material, reagents and radiolabeled materials

Phosphatidylcholine, sphingomyelin, triacylglycerol, cholesterol ester and cholesterol were purchased from Amanita Polar Lipids and Sigma Chemical Company. [Methyl-^{3}H]-choline (specific activity, 1000 mCi/ml) and [$^{1,2-3}$H]cholesteryl oleate (50 Ci/mmol) were purchased from Amersham Canada. Cholesterol oxidase, peroxidase, cholesterol esterase and reagents for protein determination were from Sigma Chemical Company. Paragon Electrophoresis System was from Beckman.

2. Cell culture material and the preparation of cell lines

Mouse peritoneal macrophages were obtained from peritoneal lavage as described (Chapter 2). The cell number was adjusted to 2×10^4 cell/ml/dish. The cells were seeded in 3.5-cm small dishes and incubated with 20% FBS RPMI 1640 cell culture medium at 37 °C for two hours. After incubation, the cells grew on the dish as a cell monolayer. The monolayer was washed with medium three times and loaded with LDL containing labeled cholesterol ester.

Smooth muscle cells were obtained from entire aorta of a Sprague-Dawley rat as described, for detail see the Chapter 2.

MEM, RPMI 1640 medium and penicillin plus streptomycin (powder) were purchased from Gibco laboratory. Cell culture dishes were from Becton Dickson and Company. FBS and BSA were purchased from Sigma Chemical Company. Trypsin was from Difco Laboratory.

3. Preparations of modified lipoproteins, apolipoprotein and microemulsion

3.1. Preparations of HDL, LDL and apolipoprotein

HDL and LDL were isolated from fresh human plasma by sequential ultracentrifugation in Beckman ultracentrifuge at 50,000 rpm, 4 °C. NaBr was used to adjusted the densities. NEM was used to inhibit the activity of plasma LCAT. HDL and LDL were obtained in NaBr solution at density ranges of 1.063-1.21 and 1.006-1.063 g/ml, respectively. The lipoprotein solutions were dialyzed against 12 L 10 mM sodium phosphate buffer containing 0.15 M NaCl at 4 °C, pH 7.4 and stored in the same solution under argon. The purity of lipoproteins were confirmed by electrophoresis in Beckman Paragon Lipo Gel. The mass ratios of lipid and protein in HDL were: phospholipid 27%; TG 3.7%; total cholesterol 14.8%; free cholesterol 3.4%; and protein 50%. The ratios in LDL were: phospholipids 15%; TG 9.7%; total cholesterol 39.8%; free cholesterol 8.7% and protein 26.6%.

ApoA-I was isolated from the fresh human HDL preparation. HDL was delipidated with ethanol/ether (2/3 v/v) and then apoA-I was isolated by DEAE-cellulose ion-exchange chromatography in 6 M urea (Tajima, et al. 1983). The preparation was dialyzed against PBS at 4 °C and stored at -72 °C under argon. Apolipoprotein composition and protein concentration were determined with polyacrylamide gel in sodium dodecyl sulfate (Laemmli, 1970) and with Lowry's method.

3.2. Preparation of modified LDLs

Details were mentioned in the previous chapter. Fresh human blood was taken with 0.2 mg/ml of EDTA and then the plasma was obtained by centrifugation. DTNB, garamycin and aprotinin were added to plasma and stirred in ice bath for 1 hour. The plasma mixture was spanned in 50,000 rpm for 20 hours to separate LDL and VLDL from the mixture. After centrifugation the top part was discarded (VLDL). The remaining fractions were collected and incubated with microemulsion at 37 °C for 2 days. The microemulsions were made through sonication of [^{3}H]-cholesterol oleate (600 µCi/0.6 ml) and phosphatidylcholine (6 mg) as described in Chapter 2. The labeled LDL was either acetylated or cationized by the method described by Basu et al. (Haraet, al. 1991; Komaba, et al 1992; Basu, et al. 1976) and stored at 4 °C under argon. The modifications of LDL were confirmed by using 0.5% agarose gel electrophoresis. Radioactivity and protein concentration of the modified LDLs were checked. The radioactivity is over 5000 cpm/µg protein and protein concentration is about 5 mg/ml.

3.3. Preparation of microemulsion

Lipid microemulsion was prepared by using triolein and egg phosphatidylcholine (1:1 by weight) through sonication at low temperature in 10 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl. The mixture was concentrated by ultracentrifugation at 99,000 rpm in a Beckman TL100 ultracentrifuge. Sepharose CL4B column was employed to purify the microemulsion. According to enzymatic assay of TG and PC, only the peak fractions with the ratio between 1.1-1.3 for TG/PC were collected and concentrated by ultracentrifugation as the final preparation. The lipid composition and concentrations were determined with enzymatic lipid assay kits. The ratio of TG to PC in final preparation was 1.25-1. Microemulsion was stored at 4 °C under argon and used within two weeks.

4. Loading cells with radiolabeled cholesterol and choline-phospholipids

Mouse peritoneal macrophages were incubated with 1 ml 0.2% BSA RPMI 1640 medium containing acetylated LDL labeled in cholesterol oleate (50 µg LDL protein /ml) and [methyl-^{3}H]-choline (2 µCi/ml) at 37 °C for 24 h. After loading of acetylated LDL, the cells demonstrated foam-cell appear under light microscope. The cells were washed three times with medium and then incubated with lipoprotein-free RPMI 1640 medium for another 24 h. Rat aorta smooth muscle cells were loaded with radiolabeled cationized LDL (40 µg/ml) in 1 ml of MEM medium for 5 days. The cells were incubated with [methyl-^{3}H]-choline for the last 24 h. The cells were washed with MEM medium three times and then incubated with MEM medium without lipoprotein for another 24 h (Komaba, et al. 1992).

5. Incubation of cholesterol-loaded cells with various of acceptors

The cholesterol loaded macrophages and smooth muscle cells were carefully washed with cell culture medium and then incubated with various concentrations of apoA-I alone, or apoA-I plus microemulsion, HDL, LDL and microemulsion in 1ml of PRMI 1640 or MEM medium containing 2 mg/ml BSA at 37 °C for various times. After a certain period of time, the cell culture medium was removed and centrifuged at 10,000 rpm for 2 min in a Beckman Microfuge to remove the cell components.

6. Analysis of lipid efflux from cells

Lipid were extracted from the medium and cells as in the method described (Brown, M. 1980) and then the aliquot of the lipid extraction was applied on silica gel (thin layer chromatography, TLC) with a microsyringe, together with 80 µg of standard lipids. After totally dried down, the TLC plates were developed by two steps to isolate cholesterol, cholesterol ester and choline-phospholipids. The developed TLC plates were stained with iodine and each lipid fraction was scraped from the plates for radioactive counting or for further analysis.

6.1. Determination of total cholesterol

The cholesterol ester in samples were hydrolyzed by using 0.05 N NaOH at 37 ^{0}C for 2 h. The enzymatic method was employed to measure the cellular total cholesterol at excitation 321 nm and emission 407 nm. The cellular cholesterol and cholesterol ester were also determined separately with the same method in several representative samples. The specific radioactivity of both macrophages and smooth muscle cells was in good agreement and also consistent with the results of gas chromatography assay (Hara and Yokoyama, 1991). The free and esterified cholesterols in medium and cells were calculated based on specific radioactivity of cellular total cholesterol and radioactivities in each fraction on TLC, as described (Komaba, et al. 1992).

6.2. Determination of phospholipids

Extracted cellular lipids were applied onto TLC. Each phospholipid fraction was scraped from the TLC plates and lipid was eluted with organic solvents for several times. Cellular specific radioactivity of each phospholipid was determined through organic phospholipid assay (Hara and Yokoyama, 1991). The phospholipids in medium were calculated based on the specific radioactivity of each cellular phospholipid.

7. Protein determination

After the lipids had been extracted, the cell monolayer was washed and treated with 0.5 N NaOH for 2 h at room temperature and then an aliquot from each individual dish was measured by Lowry's method (Lowry, et al., 1951). BSA was used as standard.

8. Density gradient ultracentrifugation

Cell culture medium was collected and cell components were removed by centrifugation. Sucrose was used to adjust the densities and 0.5 ml of density 1.31 g/ml buffer was placed in the bottom of a polyallomer quick seal tube (3 ml), then 1.5 ml of density 1.115 g/ml buffer was overplaced, finally 1 ml of medium was covered on the very top of the tube. Disturbing the layer was avoided by supplying the buffer with a microsyringe. The samples were centrifuged in a Beckman TL 100 ultracentrifuge at 4 °C and 99,000 rpm for over 16 h. The different fractions of the sample were collected by every 100 µl through a small needle punctured at the bottom of the tubes. The radioactivity of different density fractions were counted and density of each fraction was determined. The radioactivity and the density of each peak was in agreement in several examined samples.

9. Cholesterol oxidation by extracellular cholesterol oxidase

The cholesterol-loaded macrophages and smooth muscle cells were treated with extracellular cholesterol oxidase to determine the accessibility of free cholesterol from the cell surfaces (Lange and Ramos, 1983). Cells were washed with cold PBS several times and incubated with 1% glutaraldehyde for 15 min, at 0 °C. After the incubation, the cells were washed several times with 310 mM sucrose solution and then preincubated at 37 °C for 15 min in same solution. Finally, cholesterol oxidase was added with final concentration of 3 IU/ml. The reaction was terminated by removing the medium and extracting the cellular lipids right after 3 min. The cholesterol ester, free cholesterol and cholestenone were separated through thin layer chromatography. The percent oxidation of free cholesterol to total cholesterol was determined according to the specific radioactivity in each fraction and the protein were determined in each dish for standardization. In our preliminary time course experiments, it was found that no further significant reaction was observed after 3 min, as reported by Lange and Ramos.

10. Preparations of solutions and solvents

10.1. Solvent for lipid extraction from medium

Chloroform /Methanol	2:1 (v:v)

10.2. Solvent for lipid extraction from cells

Hexane/Isopropyl alcohol	3:2 (v:v)

10.3. Solvent for Thin Layer chromatography

A. For separation of phospholipids (one plate)

Chloroform	75ml
Methanol	45ml
Acetic acid	12ml
Water	6ml

B. For separation of cholesterol (one plate)

Hexane	85ml
Diethyl ether	30ml
Acetic acid	1ml

C. For separation of cholestenone (one plate)

Pertroleum ether	80ml
Diethyl ether	20ml
acetic acid	1ml

10.4. Solution for cholesterol assay

Mixture solution (for 10ml)

Sodium phosphate buffer, pH 7	1 ml
Triton X-100	50 µl
Sodium cholate	100 µl
p-hydroxyphenylacetic	2 ml
Cholesterol oxidase	100 µl
Cholesterol esterase	100 µl
Peroxidase	100 µl

Distilled water was added to give final volume to 10 ml. Solution was made before use.

Stock solution

1). 0.5M Sodium phosphate buffer
2). 10% Triton X-100
3). 10% Sodium cholate
4). 0.4% p-hydroxyphenylacetic
5). 10 IU/ml cholesterol oxidase
6). 10 IU/ml cholesterol esterase
7). 100 IU/ml peroxidase

The solutions were stored in 4 °C.

10.5. Solutions for organic phosphate assay

Reagent A (for preparing standard tubes)

(1) 10% ascorbic acid
(2) 0.42% ammonium molybdate-4 H_2O in 1 N H_2SO_4

0.3 ml of (1) and (2) mixture (1:6) was added to different concentration of standard tubes and incubated 20 min at 37 °C for 1 hour, reading at 820 nM.

Reagent B (for samples)
(1) 10% $Mg(NO_3)\cdot 6\ H_2O$ in 95% alcohol
(2) 0.5 N HCl

0.03 ml of (1) was added to samples and ashen by flame The samples were cooled and 0.3 ml of (2) was added. The tubes were bathed in boiling water for 15 min to hydrolyze pyrophosphate in the ashing. The tubes were added 0.7 ml of mixture and incubated as described in procedure for standard tubes.

RESULTS AND DISCUSSIONS

1. Lipid efflux from smooth muscle cells

According to the model proposed, it is possible to differentiate the lipid efflux through the nonspecific exchange pathway from the apolipoprotein mediated pathway by using lipid microemulsion and apoA-I. To investigate the mechanism of resistance of smooth muscle cells to free apolipoprotein-mediated cholesterol efflux, different concentrations of lipid-free apoA-I and lipid microemulsion were employed to examine the lipid efflux in various time courses.

The results in Figure III.1 (top panel) demonstrated that cholesterol efflux reached the plateau after 24 h in all different concentrations of microemulsion groups, suggesting cholesterol efflux through this pathway may be mediated by exchange mechanism. The efflux is driven by the gradient of free cholesterol between microemulsion particles and cell membranes. When the gradient reached the equilibrium state, the cholesterol efflux is reached its equilibrium as well. On the other hand, phosphatidylcholine efflux in the observed time course continuously increased but the maximum rate of phosphotidylcholine efflux was much less than that of cholesterol, suggesting that phosphatidylcholine efflux mediated by microemulsion is slower than that of cholesterol at least within our time course.

Figure III.1 (lower panel) showed the time course of lipid efflux induced by lipid free apoA-I. It was shown that the rate of cholesterol efflux was low, as we observed in previous experiments, and phosphatidylcholine efflux was much greater than that of cholesterol in terms of lipid weight in the efflux medium. The results implied that phospholipid efflux through the free apolipoprotein-mediated pathway is faster and more efficient than that of cholesterol. Since cholesterol and phospholipid efflux induced by microemulsion and apoA-I were continuously increased during the initial 24 h of incubation, 24 hour-incubation was chosen for further observation of lipid efflux.

To investigate the characters of particles formed in free apoA-I-mediated lipid efflux, density gradient ultracentrifugation was used to isolate the particles. It was demonstrated that apoA-I-mediated lipid efflux from smooth muscle cells formed new HDL-like particles (density about 1.1-1.2 g/ml) although the particles were poor in cholesterol and rich in phosphatidylcholine, as showed in the inset of Figure III.1.

Figure III.2 demonstrated the concentration-dependent lipid efflux mediated by microemulsion, apoA-I and HDL. The panel in the top shows microemulsion-mediated lipid efflux. The kinetic profiles of cholesterol, phosphatidylcholine and sphingomyelin efflux were the same (concentration dependent and saturatable) but cholesterol efflux was much greater than that of either phospholipid. The Km of phosphatidylcholine was estimated about 8×10^{-5} M.

The middle panel shows the profile lipid efflux mediated by apoA-I. Phospholipid efflux was much greater than that of cholesterol. It agreed with the previous work that smooth muscle cells were resistant to free apolipoprotein-mediated cholesterol efflux. The lipid efflux induced by apoA-I also generated HDL-like particles, as show in Figure III.1, but cholesterol efflux from smooth muscle cells through the particle generation was much slower in comparson with that of macrophages and fibroblasts (Komaba, et al. 1992). V_{max} of cholesterol efflux to apoA-I from smooth muscle cells was only 3% of the cellular total free cholesterol and Km of apoA-I was 1.5 μg/ml. The rate of phosphatidylcholine efflux was about three times greater than that of cholesterol with approximately the same Km value. It may imply the poor availability of cholesterol to apoA-I mediated cholesterol efflux.

The bottom panel demonstrats the lipid efflux induced by HDL. The kinetic profiles were the same as that of the microemulsion, i.e., the rate of cholesterol efflux was greater than that of phosphatidylcholine. The ratio of cholesterol to PC in HDL-mediated lipid efflux was in the middle of those of the free apolipoprotein and lipid-microemulsion. This may be explained by the contribution of free apolipoprotein dissociated from HDL particles. These free apolipoproteins mediated relatively higher PC efflux and lower cholesterol efflux as shown in the middle panel of Figure III.2.

2. Lipid efflux from macrophages

Figure III.3 shows lipid efflux from macrophages. Microemulsion-induced lipid efflux from macrophages is shown in top panel. The kinetic profile was similar to that of smooth muscle cells, the rate of cholesterol efflux was much greater than that of either phospholipid. The V_{max} of cholesterol was about 25% total free cholesterol with Km about 4.5×10^{-5} M microemulsion phosphatidylcholine.

The results of lipid efflux by lipid free-apoA-I are shown in the middle panel. It was demonstrated that the new HDL-like particles were generated by apoA-I-mediated lipid efflux which consisted great amount of phospholipids and considerable amount of cholesterol. It was noticeable that lipid free apoA-I-mediated cholesterol efflux from smooth muscle cells was dramatically less (Figure III.2) compared to cholesterol efflux from macrophages. However, the phospholipid efflux from both cell was very similar in terms of the Vmax, therefore, the ratio of cholesterol to phospholipid in macrophages was three to four times higher than that in smooth muscle cells. It was suggested that in smooth muscle cells only cholesterol but not phospholipid was poorly available for apoA-I-mediated lipid efflux.

The lower panel presented the HDL-mediated lipid efflux from macrophages. The results demonstrated that the kinetic profile of the lipid efflux was similar to that of smooth muscle cells.

3. The ratio of phospholipid/cholesterol in lipid efflux

To investigate the mechanism of the resistance of smooth muscle cell to free apolipoprotein-mediated cholesterol efflux, we tried to compare the lipid efflux from both cells and differentiate the lipid efflux through both exchange and apolipoprotein-mediated pathways. All results from the experiments were summarized in Table III.1 as the weight ratio of phospholipid to cholesterol.

The ratios in microemulsion groups were very similar in both cells, 0.13-0.28 for smooth muscle cells and 0.10-0.29 for macrophages respectively. The lipid efflux through the nonspecific exchange pathway seemed to be similar between both cell types in terms of the kinetic profile of the lipid efflux. Therefore, this implied that the nonspecific exchange pathway may not be involved in the resistance of smooth muscle cells to apolipoprotein-mediated cholesterol efflux. The ratio of PC to cholesterol for LDL-mediated lipid efflux in both cells was similar too, indicating the contribution of LDL to lipid efflux just like lipid microemulsions.

The ratios of phospholipid to cholesterol were very different in apoA-I groups between both cells. As shown in Table III.1 the ratios in smooth muscle cells were much greater than that in macrophages. This was because considerably less cholesterol efflux occurred from smooth muscle cells with a similar amount of phospholipid efflux from both cells. From the comparison of cholesterol and phospholipid efflux from both cells, it was indicated that the low rate of cholesterol efflux from smooth muscle cells was not because of low reactivity of smooth muscle cells with apoA-I since phospholipid efflux from both cells was quite similar. However, the apolipoprotein-mediated lipid efflux from smooth muscle cells resulted in the generation of HDL-like particles in medium also although the particles were phospholipid-rich and cholesterol-poor.

The ratios of phospholipid to cholesterol in HDL-mediated lipid efflux were in between those of apoA-I and microemulsion.

The ratio was higher than that of microemulsion and lower than that of apoA-I. In addition, the ratios in smooth muscle cells were higher than that of macrophages. It was assumed that apolipoproteins could dissociate from and associate with HDL particle surfaces. It allowed apolipoproteins (dissociated from HDL) to contribute to lipid efflux as free apolipoproteins, inducing relatively more phospholipid efflux than cholesterol efflux. This can also explain the higher ratios of PC/cholesterol in HDL-mediated lipid efflux from smooth muscle cells.

4. Contribution of apolipoprotein to lipoprotein mediated lipid efflux

To study the contributions of the lipid-bound and lipid-free apolipoproteins to lipid efflux, we used a system with a constant concentration of microemulsion and an increasing concentration of apoA-I. As shown in the Figure III.4, cholesterol efflux increased as a sigmoid function of apoA-I concentration in medium. This kinatic profile of cholesterol efflux was consistent with the model of free apoA-I but not lipid-bound apoA-I-mediated cholesterol efflux (Hara and Yokoyama, 1992). In lower concentration ranges of apoA-I, the efflux of phosphatidylcholine and cholesterol were almost similar to control (microemulsion alone). However, as apoA-I concentration increased in medium, both phosphatidylcholine and cholesterol efflux started to increase and eventually reached the maximum. Phosphatidylcholine efflux increased by about 2.5-3 times but cholesterol efflux increased only by 30%-50%. As a result, the ratio of phosphatidylcholine to cholesterol in lipid efflux also increased as apoA-I increased in medium (bottom panel). Thus, the data are consistent with the model that lipid-free apoA-I-mediated lipid efflux was composed of higher phospholipid and lower cholesterol efflux. We assume that at a lower apoA-I concentration, apoA-I associated with microemulsion particles and did not contribute to lipid efflux; as apoA-I increased beyond the concentration required to saturate the microemulsion surface, the concentration of free apoA-I in the medium increased, therefore the manner of the lipid efflux was similar to that of free apoA-I (more phosphatidylcholine and less cholesterol efflux). Hence, this suggested only free apoA-I not lipid-bound apoA-I was responsible for lipid efflux.

In Figure III.5, LDL was used instead of microemulsion with increasing concentration of apoA-I. It was indicated that lipid efflux increased linearly rather than sigmoidally as apoA-I increased in medium in the initial phase. PC efflux was enhanced 3 times at the maximum but cholesterol efflux increased only 50%. This is again consistent with the results of free apoA-I-mediated lipid efflux from SMC (higher PC efflux and lower cholesterol efflux). It is likely that apo B covers almost all of the LDL surface and prevents apoA-I from association with LDL surface, so that apoA-I contributed to LDL-mediated lipid efflux only as free apoA-I. Therefore even in the lower concentration, apoA-I increased the ratio of phospholipid to cholesterol in LDL-mediated lipid efflux comparing to lipid efflux in LDL alone.

5. Probing surface cholesterol

In order to study the possible mechanism of resistance of smooth muscle cells to apoA-I-mediated cholesterol efflux, the accessibility of cellular cholesterol was assessed by using extracellular cholesterol oxidase as a probe. Table III.2 demonstrats the results of surface cholesterol oxidation and the V_{max} rate of cellular free cholesterol efflux from smooth muscle cells and macrophages. Under our experimental condition, cellular free cholesterol oxidation by cholesterol oxidase was higher in macrophages than that in smooth muscle cells. This method may detect the accessible free cholesterol on the cell membrane which may directly be available for non-specific exchange pathway. Therefore, we presume that the slightly lower Vmax for non-specific efflux induced by lipid-microemulsion was a reflection of the somewhat lower accessibility of free cholesterol in smooth muscle cells. However, this small difference cannot explain the large difference in apoA-I-mediated cholesterol efflux between both cells. There are two possibilities for the failure of the oxidase to detect the cholesterol pool for apoA-I-mediated cholesterol efflux: one is that there is a special pool for apolipoprotein-mediated cholesterol efflux while the cholesterol oxidation method can only detect the free cholesterol pool available to the non-specific exchange pathway. The other is that the cholesterol oxidation method can only determine the content of free cholesterol on the accessible cell surface in a certain time course but can not reflect the exchange rate of intracellular cholesterol and plasma membrane cholesterol pools. This exchange rate may also affect the cholesterol efflux by supplying intracellular cholesterol to the membrane surface.

From the results, we conclude the following: (1) Cellular cholesterol in both macrophages and smooth muscle cells is exchangeable with an extracellular pool. (2) The availability of cholesterol for apolipoprotein-mediated lipid efflux is extremely poor in smooth muscle cells though (3) there are no big difference in the reaction between cell and apoA-I as demonstrated by a similar rate of phospholipid efflux from both cells. (4) Phospholipid efflux mediated by microemulsion and HDL (through the nonspecific pathway) is much slower than cholesterol efflux through apolipoprotein-mediated efflux pathway from both cells.

These findings provide more evidence that there are at least two pathways for cellular cholesterol efflux. One pathway is the nonspecific exchange between the cell surface and the extracellular lipid surface, mostly the lipoprotein surface in vivo, by diffusion through the aqueous phase or by collision between membranes. In this pathway, specific interaction between the acceptor lipoproteins and cell surface is unlikely to be involved (Karlin et al., 1987; Hara & Yokoyama, 1992). The other pathway is net lipid removal by apolipoproteins and generation new preb HDL-like particles with cellular lipids (Hara, et al. 1991, 1992; Hara, et al., 1992; Komaba, et al., 1992). The physiological relevance of this reaction is justified by the low Km value for the apolipoprotein concentration such as 1/1500 of plasma apoA-I for fibroblasts and smooth muscle cells and 1/500 for macrophages. This observation is consistent with the data that cellular cholesterol appears in preb-HDL fraction prior to other lipoproteins in plasma in vitro (Castro, et al. 1988; Miida, et al., 1990). Since nonspecific exchange of phospholipid is much slower than cholesterol, these two pathways are distinguishable by the relative ratio of phospholipid to cholesterol in lipid efflux. It is proved by experimental data that apoA-I facilitated more phospholipid efflux than that of cholesterol from smooth muscle cells to microemulsion (Fig. 4).

Cholesterol was poorly available in the lipid efflux mediated by lipid-free apolipoproteins from smooth muscle cells. The results from the cellular cholesterol oxidation demonstrated that the lower

Vmax of nonspecific cholesterol efflux may be caused by the lower accessibility of the cellular free cholesterol pool, but the poor availability of cholesterol in apolipoprotein-mediated lipid efflux from smooth muscle cells was very unique and specific.

We postulate a few possible mechanisms for the poor availability of cholesterol for apolipoprotein-mediated lipid efflux. The first possibility is that the smooth muscle cell surface contains less cholesterol than other cell surface, but because of rapid exchange of cholesterol between pools, the Vmax of the efflux by the exchange mechanism may not be affected too much by the low cholesterol content on the surface. This is consistent with the data that Vmax of microemulsion-mediated cholesterol efflux is slightly lower in smooth muscle cells than that in macrophages. Lower content of surface cholesterol may have more effect on apolipoprotein-mediated cholesterol efflux since cholesterol efflux from this pathway may involve the direct interaction between cells and apolipoprotein to generate new HDL-like particles by removing membrane lipid. Therefore, the lipid composition of the surface may be more directly reflected in the lipid efflux by this mechanism. The second possibility is that there is a specific membrane cholesterol pool available for the apolipoprotein-mediated lipid efflux or that there are a specific mechanism by which cholesterol is incorporated into newly generated HDL-like particles. Mahlberg and Rothblat (1992) reported that there are two distinguishable kinetic pools of cholesterol on the cell surface for the nonspecific exchange reaction. The mechanism for creating different cholesterol pool in the membrane is unknown. One of possibility is that cholesterol may associate with other specific membrane component, such as sphingomyelin, and lead to restriction of exchangeability of cholesterol with other membrane. To distinguish these two possibilities, it is necessary to measure cholesterol on cell surface. There is also possibility of specific protein(s) which modulate(s) lipid efflux. The protein(s) may play different role in both cells.

The poor cholesterol efflux mediated by apolipoprotein from smooth muscle cells implies that atherosclerotic vascular lesions in the stage predominant in smooth muscle cells are very resistant to regression. This is not because the cells are less reactive with apolipoproteins but because cellular cholesterol is not available by whatever reason for this reaction. Therefore, it may be possible to modulate cellular cholesterol metabolism in smooth muscle cells in order to create a cellular cholesterol pool reactive with the apolipoprotein mediated lipid efflux and to make smooth muscle cell-dominated vascular lesions regressive.

REFERENCES

Aviram, M., Bierman, E. L., and Oram, J. F. High density lipoprotein stimulates sterol translocation between intracellular and plasma membrane pools in human monocyte-derived macrophages. J. Lipid. Res. 1989; 30: 65-76

Basu, S. K., and Goldstein, J. L. Degradation of cationized LDL and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. Proc. Natl. Acad. Sci. U. S. A. 1976; 73: 3178-3182

Brown, M. S., Ho, Y. K., and Goldstein, J. L. The cholesterol ester cycle in macrophage foam cells. J. Biol. Chem. 1980; 255: 9344-9352

Burns, C. H., and Rothblat, G. H. Cholesterol extraction by tissue culture cells: effect of serum lipids. Biochim. Biophys. Acta. 1969;176: 616-625

Castro, G. R., and Fielding, C. J. Early incorporation of cell-derived cholesterol into pre-b-migrating high density lipoprotein. Biochemistry. 1988; 27: 25-29

Hara, H. and Yokoyama, S. Interaction of free apolipoproteins with macrophages. J. Biol. Chem. 1991; 266: 3080-3086

Hara, H. and Yokoyama, S. Role of apolipoprotein in cholesterol efflux from macrophages to lipid microemulsion: proposal of a putative model for the pre-b high density lipoprotein pathway. Biochemistry. 1992; 31: 2040-2046

Ho, Y. K., Brown, M. S., and Goldstein, J. L. Hydrolysis and excretion of cytoplasmic cholesterol ester by macrophages stimulation by high density lipoprotein and other agents. J. Lipid Res. 1980; 21: 391-398

Johnson, W. J., Bamberger, M. J., Latta, R. A. The bidirection flux of cholesterol between cells and lipoproteins. J. Biol. Chem. 1986; 261: 5766-5776

Johnson, W. J., Mahlberg, F. H., Chako, G. K. et al. The influence of cellular and lipoprotein cholesterol contents on the flux of cholesterol between fibroblasts and high density lipoprotein. J. Biol. Chem. 1988; 263: 14099-14106

Karlin, J. B., Johnson, W. J., Benedict, C. R. Cholesterol flux between cells and high density lipoprotein. J. Biol. Chem. 1987; 262: 12557-12564

Komaba, A., Li, Q. Hara, H., and Yokoyama, S. Resistance of smooth muscle cells to assembly of high density lipoproteins with extracellular free apolipoproteins and to reduction of intracellularly accumulated cholesterol. J. Biol. Chem. 1992; 267: 17560-17566

Lange, Y. and Ramos, B. V. Analysis of the distribution of cholesterol in the intact cell. J. Biol. Chem. 1983; 258: 15130-15134

Lowry, O. H., et al. Protein measurement with the folin phenol reagent. J. Biol. Chem. 1951; 193: 265-275

Mahlberg, F. H. and Rothblat, G. H. Cellular cholesterol efflux. J. Biol. Chem. 1992; 267: 4541-4550

McKnight, G. L., Reasoner, J., Gilbert, T. et al. Cloning and expression of a cellular high density lipoprotein-binding protein that is up-regulated by cholesterol loading of cells. J. Biol. Chem. 1992; 267: 12131-12141

Miida, T. Fielding, C. J., and Fielding, P. E. Mechanism of transfer of LDL-derived free cholesterol to HDL subfraction in human plasma. Biochemistry. 1990; 29: 10469-10474

Nishikawa, O., Yokoyama, S. and Yamamoto, A. A method for incorporation of radiolabeled cholesterol ester into human plasma low-density-lipoproteins: preparation of trace substrate for cholesterol ester transfer reaction between lipoproteins. J. Biol. Chem. 1986; 99: 295-301

Rothblat, G. H., and Philips, M. C. Mechanism of cholesterol efflux from cells. J. Biol. chem. 1982; 257: 4775-4782

Savion, N. and Kotev-Emeth, S. Cholesterol efflux from and high density lipoproteins binding to cultured bovine vascular endothelial cells are higher with vascular smooth muscle cells. Eur. J. Biochem. 1989; 183: 363-370

Slotte, J. P., Oram, J. F., and Bierman, E. L. Binding of high density lipoproteins to cell receptors promotes translocation of cholesterol from intracellular membranes to the cell surface. J. Biol. Chem. 1987; 262: 12904-12907

Stien, O., Vanderhoek, J., and Stein, Y. Cholesterol content and sterol synthesis in human skin fibroblasts and rat smooth muscle cells, and fibroblasts. Biochim. Biophys Acta. 1976; 431: 347-358

Tabas, I., and Tall, A. R. Metabolism of association of HDL3 with endothelial cells, smooth muscle cells, and fibroblasts. J. Biol. Chem. 1984; 259: 13897-13905

Tajima, S., Yokoyama, S. and Yamamoto, A. Effect of particle size on association of apolipoproteins with lipid. J. Biol. Chem. 1983; 258: 10073-10082

Werb, Z., and Cohen, Z. A. Cholesterol metabolism in the macrophages. J. Exp. Med. 1971; 134: 1545-1590

Figure III.1. Time course of lipid efflux from smooth muscle cells. The cells were loaded with cholesterol by incubating with cationized LDL as described in MATERIALS AND METHODS, giving the initial conditions for the lipid efflux as cell protein 167µg/ml, phosphatidylcholine 1.76µg/ml, sphingomyelin 1.40µg/ml, and free and esterified cholesterol 1.54 and 1.75µg/ml, respectively. Cellular lipid efflux was induced (solid lines, cholesterol; broken lines, phosphatidylcholine) by lipid microemulsion (upper panel) 100 µM (○), 200 µM (●), and 400 µM (△) as phosphatidylcholine concentration in the medium and by lipid-free apoA-I (lower panel) 1µg/ml (○), 2µg/ml (●), 5µg/ml (△) and 10µg/ml (▲) in the medium. Inset of the lower panel is the result of density gradient ultracentrifugation analysis for phosphatidylcholine in the medium of smooth muscle cells after incubation with 10µg of apoA-I for 24 h. Lipid was extracted from each fraction and separated by thin layer chromatography to obtain the radioactivity in phosphatidylcholine.

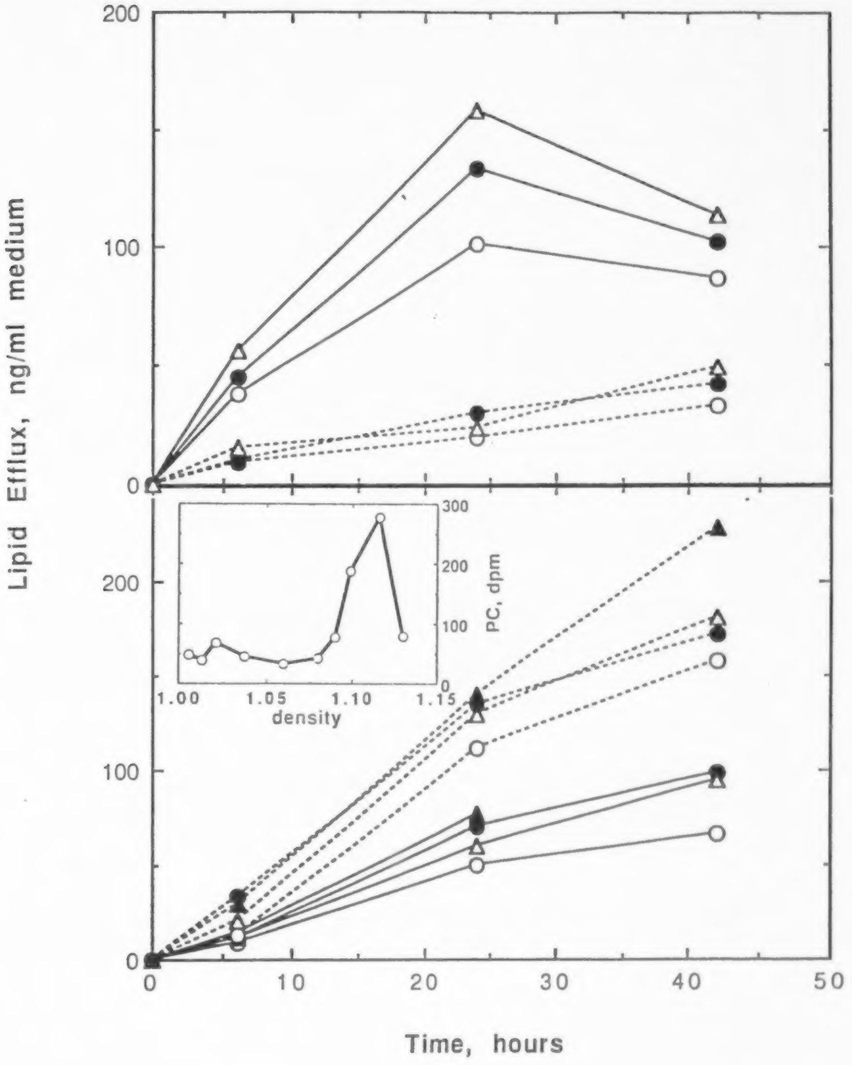

200
100
0
Lipid Efflux, ng/ml medium
200
100
0
0
10
20
30
40
50
Time, hours
300
200
100
0
PC, dpm
1.00
1.05
1.10
1.15
density

Figure III.1

Figure III.2. Cellular lipid efflux from smooth muscle cells. The cells were loaded with cholesterol by incubated with cationized LDL as described in MATERIALS AND METHODS, giving the initial condition for lipid efflux as cell protein 272µg/ml, phosphatidylcholine 1.62µg/ml, sphingomyelin 0.92µ/ml, and free and esterified cholesterol 2.03 and 1.01 mg/ml, respectively. The efflux of cellular cholesterol (○), phosphatidylcholine (●) and sphingomyelin were measured in the medium induced by lipid microemulsion (top panel), lipid-free apoA-I (middle) and HDL (bottom).

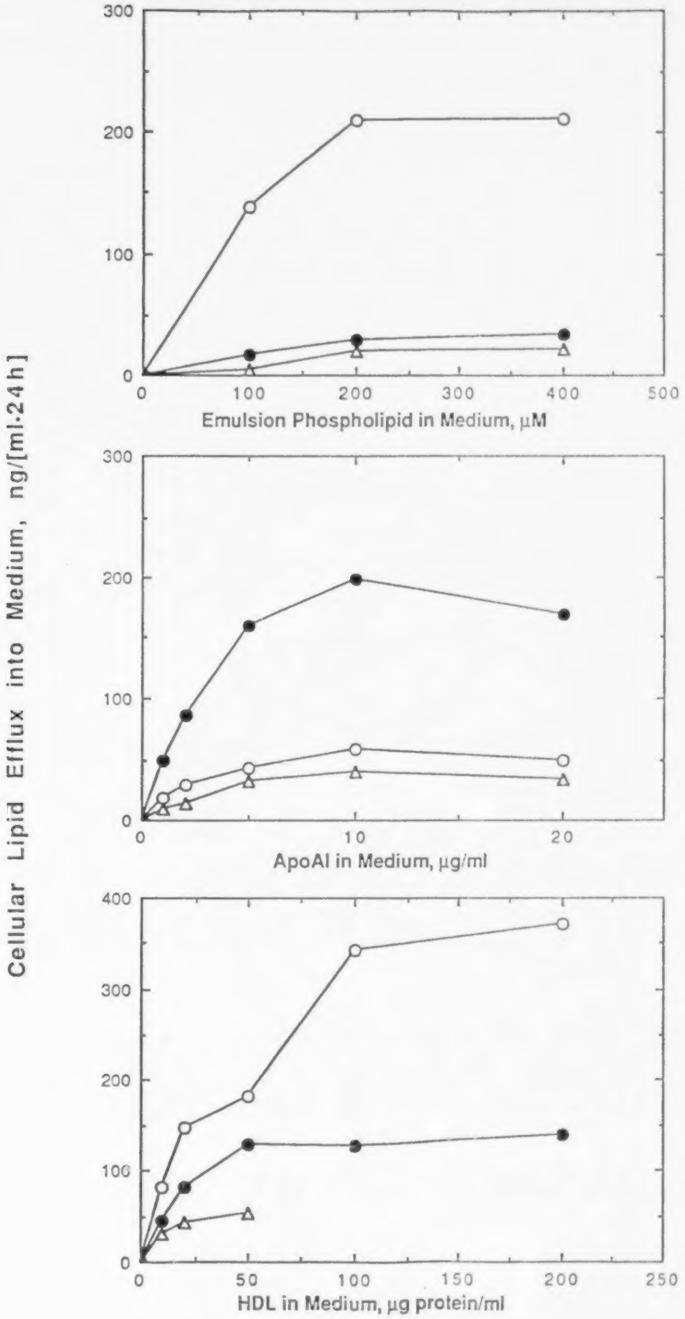

Cellular Lipid Efflux into Medium, ng/[ml·24h]
300
200
100
0
0
100
200
300
400
500
Emulsion Phospholipid in Medium, µM
300
200
100
0
0
10
20
ApoAI in Medium, µg/ml
400
300
200
100
0
0
50
100
150
200
250
HDL in Medium, µg protein/ml

Figure III.2

Figure III.3. Cellular lipid efflux from macrophages. The cell were loaded with cholesterol by incubated with acetylated LDL as described in MATERIALS AND METHODS, giving the initial condition as cell protein 26 μg/ml, phosphatidylcholine 0.80μg/ml, sphingomyelin 0.5μg/ml, and free and esterified cholesterol 1.3m and 2.71μ/ml, respectively. The efflux of cellular cholesterol (○), phosphatidylcholine (●) and sphingomyelin (Δ) were measured in the medium induced by lipid microemulsion (top), lipid-free apoA-I (middle) and HDL (bottom).

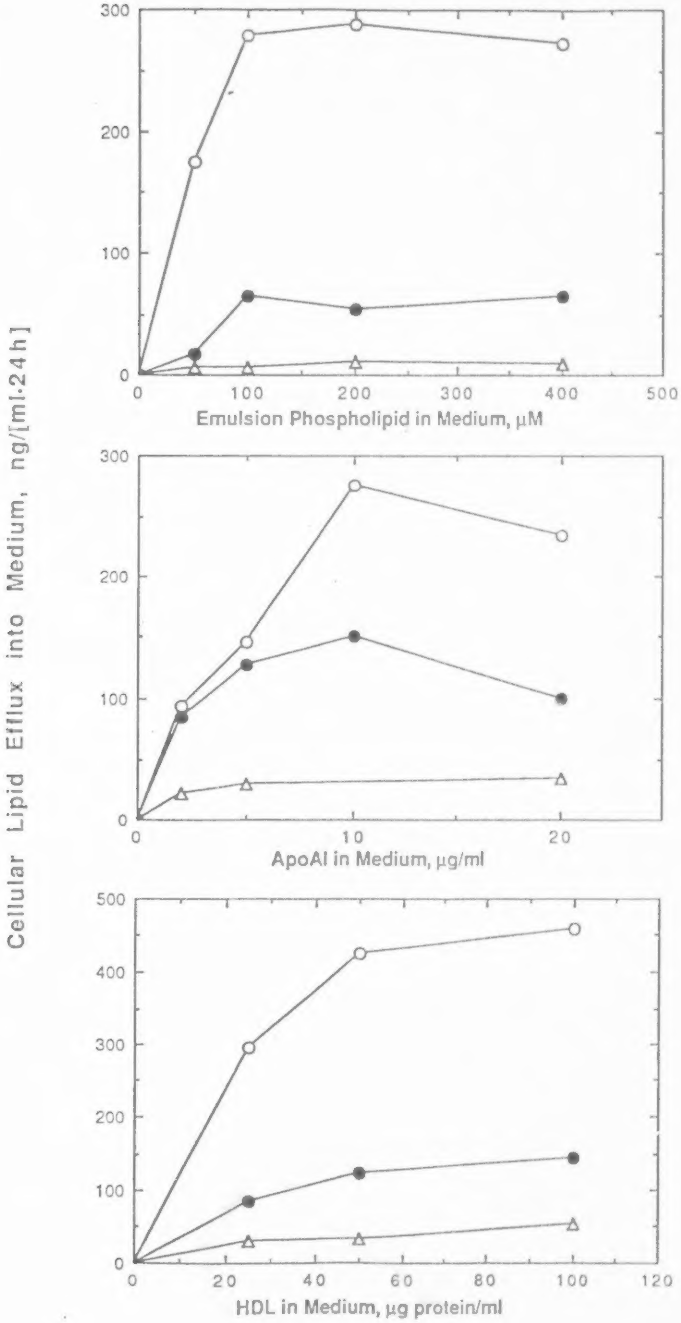

Cellular Lipid Efflux into Medium, ng/[ml·24 h]
300
200
100
0
0
100
200
300
400
500
Emulsion Phospholipid in Medium, μM
300
200
100
0
0
10
20
ApoAI in Medium, μg/ml
500
400
300
200
100
0
0
20
40
60
80
100
120
HDL in Medium, μg protein/ml

Figure III. 3

Figure III.4. The effect of apoA-I on the lipid efflux from smooth muscle cells induced by lipid microemulsion. The initial condition is the same as that in Figure III.1. Lipid-free apoA-I was added to lipid microemulsion, 200 μM phosphatidylcholine in the medium. The upper panel shows the efflux of cholesterol (○) and phosphatidylcholine (●), and the lower panel shows the ratio of phosphatidylcholine to cholesterol (○). The error bars indicated standard error of three experimental points.

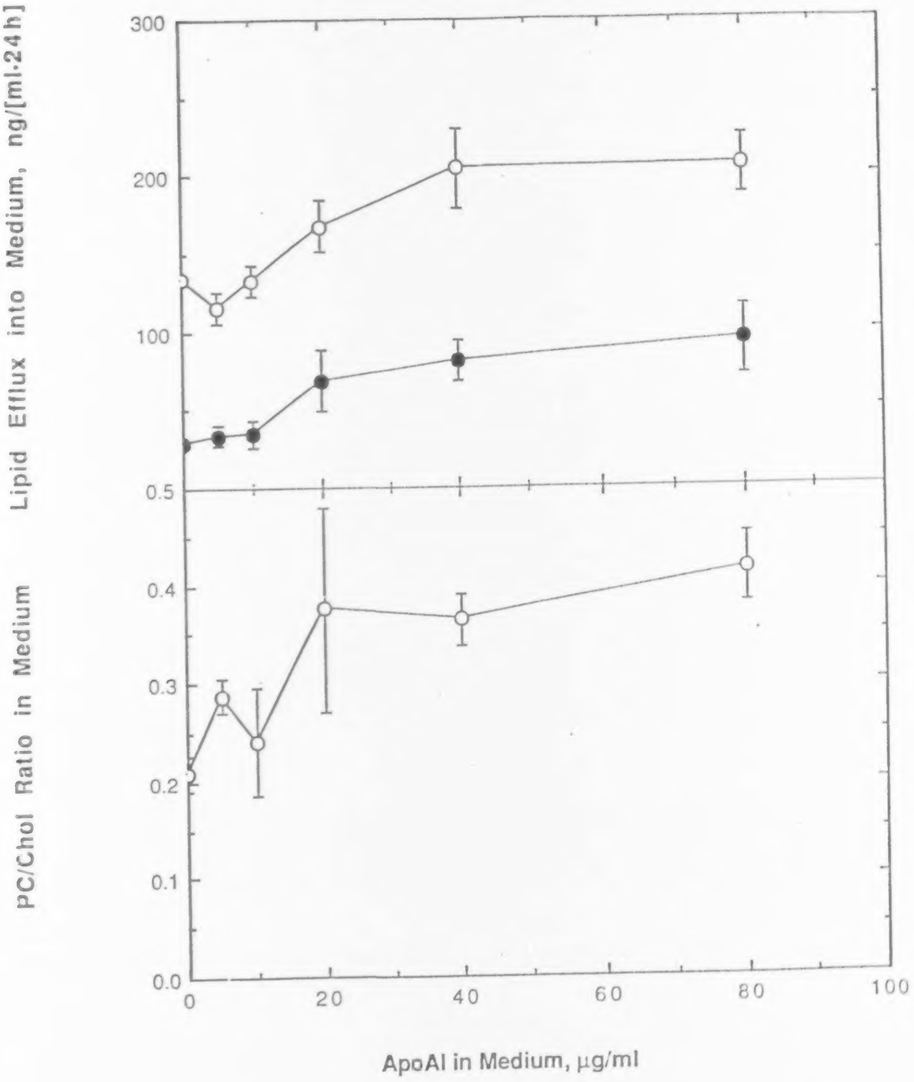
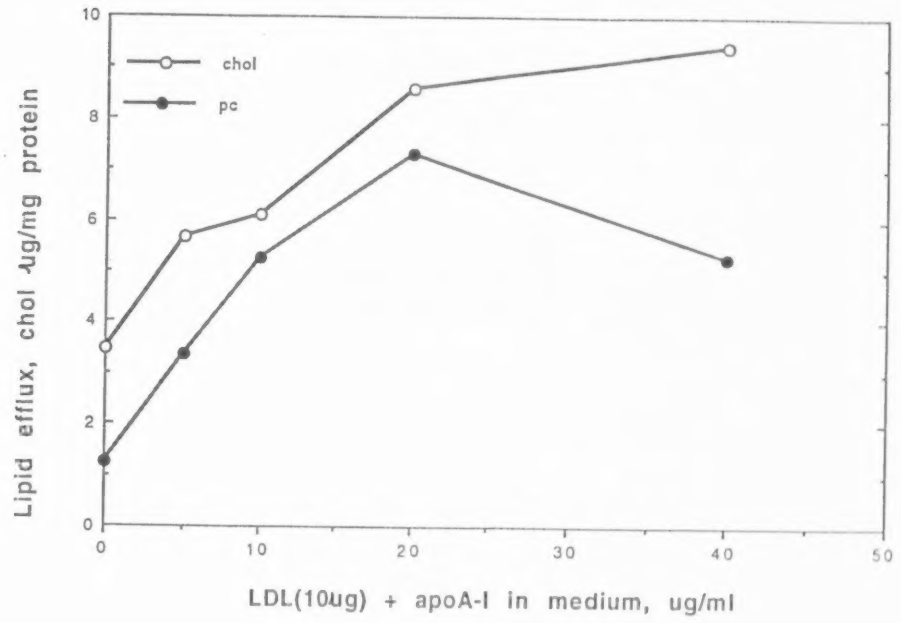

Lipid Efflux into Medium, ng/[ml·24h]
300
200
100
0.5
0.4
0.3
0.2
0.1
0.0
PC/Chol Ratio in Medium
0
20
40
60
80
100
ApoAI in Medium, µg/ml

Figure III.4

Figure III.5. Contribution of apoA-I to LDL mediated lipid efflux. The initial conditions are the same as that in Figure III.1. Different concentration of lipid-free apoA-I were added to LDL containing medium (10 μg/ml) and incubated for 24 h. The medium were collected and cholesterol efflux (○) and phospholipid (●) efflux were measured. The lipid efflux were standardized by protein per dish.



10
8
6
4
2
0
Lipid efflux, chol ug/mg protein
chol
pc
0
10
20
30
40
50
LDL(10ug) + apoA-I in medium, ug/ml

Figure III.5

Table III.1 Phospholipid/cholesterol ratio in lipid efflux from cells. Efflux of cellular free cholesterol, phosphatidylcholine (PC) and sphingomyelin (SM) induced by apoA-I, lipid microemulsion and lipoproteins were measured in the medium according to the method described in MATERIALS AND METHODS, and the ratios of PC and SM to cholesterol were calculated as weight/weight. For smooth muscle cells, Figure III.1 corresponds to apoA-I experiment #3 and microemulsion #3, and Figure III.2 corresponds to apoA-I #2, microemulsion #2 and HDL #1. For macrophage, Figure III.3 corresponds to apoA-I #2, microemulsion #3 and HDL #1. Data represent mean for 3 to 5 experimental points in each series.

Table III.1 Phospholipid/cholesterol ratio in lipid efflux from cells

Efflux Inducer		Smooth Muscle Cells			Macrophages	
		PC/Chol	SM/Chol		PC/Chol	SM/Chol
ApoA-I	#1[a]	2.10 ± 0.42	0.43 ± 0.12	#1	0.43 ± 0.17	0.15 ± 0.05
	#2	3.20 ± 0.39	0.65 ± 0.20	#2	0.69 ± 0.20	0.24 ± 0.10
	#3	2.09 ± 0.45				
	#4	6.02 ± 1.10				
Emulsion	#1	0.13 ± 0.04	0.013 ± 0.004	#1	0.29 ± 0.12	0.16 ± 0.08
	#2	0.15 ± 0.014	0.077 ± 0.030	#2	0.10 ± 0.01	0.036 ± 0.017
	#3	0.28 ± 0.09		#3	0.18 ± 0.03	0.031 ± 0.009
	#4	0.27 ± 0.11		#4	0.23 ± 0.05	0.105 ± 0.032
LDL	#1	0.17 ± 0.04		#1	0.14 ± 0.03	0.086 ± 0.023
HDL	#1	0.83 ± 0.08	0.45 ± 0.12	#1	0.30 ± 0.012	0.100 ± 0.016
	#2	0.90 ± 0.26				

PC, Phosphatidylcholine; SM, Sphingomyelin; Chol, Cholesterol

[a] Experiment series number (#).

Table III.2. Accessibility to cellular cholesterol by extracellular probes. The values are percent of the initial cellular free cholesterol pool.

	Smooth Muscle Cells	Macrophages
V_{max}/cellular free cholesterol[a]		
Microemulsion	18.2 ± 1.6 (3[b])	43.3 ± 12.4 (3)
HDL	36.9 ± 2.4 (3)	72.5 ± 8.4 (3)
apoA-I	3.5 ± 0.5 (3)	35.9 ± 5.9 (3)
Cholesterol oxidation[c]	27.3 ± 2.3 (6)	42.2 ± 4.8 (6)

[a] Apparent V_{max} rate of cellular free cholesterol efflux per 24 hours standardized as percent of the initial cellular free cholesterol pool.

[b] Number of experiments.

[c] Percent oxidation of cellular free cholesterol in 3 minutes by extracellular cholesterol oxidase probes.

Chapter IV.

Effect of Cellular Factors on Apolipoproptein-Mediated Cholesterol Efflux

INTRODUCTION

Cholesterol is synthesized mainly in the liver and transferred to peripheral cells in the form of lipoprotein (LDL). The cholesterol level in the cell membrane is well regulated by several mechanisms which control the cholesterol influx, esterification and efflux. The regulation of cholesterol uptake and esterification has been elucidated recently. However, the cellular regulatory mechanism of cholesterol efflux is not yet fully understood.

In physiological conditions, the peripheral cells which can deposit large amount of cholesterol ester called foam cells. These foam cells appear in early atherosclerotic lesions, as well as in mature plaques. Both smooth muscle cells and macrophages can be transformed into foam cells by loading cholesterol (Ross, 1993). The accumulated cholesterol has to be transported from these cells to the liver for further metabolism. This process of transporting cellular cholesterol from peripheral cells to the liver is termed reverse cholesterol transport. It may play an important role in regression and prevention of atherosclerosis. Cholesterol efflux from the cells is the first step in this process. Therefore, the mechanism of cholesterol efflux has been intensively studied by many groups.

It is well known that cellular free cholesterol is exchangeable with extracellular cholesterol (Phillips, et al. 1987). In this process, cholesterol molecules diffuses through an aqueous phase from the higher gradient to the lower gradient. Therefore, net cholesterol efflux from cells only occurs in cholesterol enriched membranes. The rate-limiting step in this process is the rate of cholesterol molecule desorption from the cell membrane (Rothblat, et al. 1982; Johnson, et al. 1986; Karlin, et al. 1987).

HDL is believed to be the major physiological acceptor of cellular cholesterol (Gordon, et al. 1977). The kinetics of HDL-mediated cholesterol efflux are consistent with this diffusion model. Therefore, physical properties of HDL, such as lipid composition, play a more important role than biological factors ie. surface apolipoprotein component and cell membrane protein in cholesterol efflux (William, et al. 1991). However, this nonspecific diffusion model can not explain HDL-mediated net cholesterol efflux nor estabolish any connection between the cholesterol efflux and cellular regulatory mechanism.

On the other hand, cholesterol efflux also involves HDL-binding proteins on cell surfaces (Slotte, et al. 1987). First, the number of binding sites of these proteins is up-regulated by loading cells with cholesterol (McKnight, et al. 1992), suggesting the function of these proteins may involve the removal of excess cholesterol. Second, binding of HDL to the proteins reportedly activates protein kinase C and triggers the translocation of intracellular cholesterol to the plasma membrane (Oram, et al. 1990; Mendez, et al. 1991). This indicates that cholesterol efflux may be a cellularly regulated process.

In our lab, previous studies showed that free apolipoproteins can mediate net cholesterol efflux from macrophage-derived foam cells by generating pre-HDL particles (Hara, et al. 1991). This reaction requires only a low concentration of apolipoproteins (1/500 plasma concentration), thus implicating the biological relevance of this pathway and its potental role in atherosclerosis regression and HDL formation. Smooth muscle cells (SMC) are resistant to apolipoprotein-mediated cholesterol efflux (Komaba, et al. 1992; Li, et al. 1993), chapter 2 and 3. This resistance is not due to the poor interaction between SMC and apolipoproteins but due to the poor availability of cholesterol from SMC (Li, et al. 1993), as described in chapter 3. As reported, apolipoproteins (apoA-I and A-II) on HDL can induce cholesterol efflux from the plasma membrane by stimulating translocation of cholesterol from intracellular pools (Oram, et al. 1991). Therefore, it is possible that poor cholesterol efflux from SMC may be caused by poor cholesterol content in the membrane. This may indicate the poor cholesterol transfer from the intracellular pool to the plasma membrane. The poor cholesterol transfer may involve cellular mechanisms, ie. cholesterol esterification, hydrolysis, and translocation.

The present study explores cellular factors which may be involved in apolipoprotein-mediated cholesterol efflux. To address this issue, we treat cholesterol-loaded SMC with Macrophage Colony-Stimulating Factor (M-CSF) and Platelet-derived Growth Factor (PDGF). PDGF can induce c-fms (gene carrying M-CSF receptor) expression in vascular SMC. Furthermore, SMC can respond to M-CSF and convert to macrophage-like cells (Inaba T. et al., 1992_1 and 1992_2). M-CSF is able to stimulate cholesterol metabolism in vivo and enhance cholesterol efflux from cholesterol-loaded human monocyte-derived macrophages in vitro (Inoue, et al. 1992 and Shimano, et al. 1990). Therefore, it is expected that the resistance of SMC to apolipoprotein-mediated cholesterol efflux can be changed when SMC are converted into macrophage-like stage. SMC are also treated with phorbol ester which is activator of Protein kinase C (PKC) to investigate the involvement of cellular factors in cholesterol efflux.

The results indicate that apoA-I-mediated, rather than HDL-mediated, cholesterol efflux is enhanced when SMC are treated with M-CSF and PDGF plus PMA. It suggests that the mechanism for apolipoprotein-mediated cholesterol efflux is different from that of HDL and it also involves a cellular regulatory process.

MATERIALS AND METHODS

1. Lipoproteins and apolipoproteins

HDL and LDL were isolated from fresh human plasma as densities 1.063-1.21 g/ml and 1.006-1.063 g/ml, respectively. The lipoprotein preparations were dialyzed against 10 mM phosphate buffer as described before. The lipoprotein purity was confirmed by criteria of electrophoretic mobility in agarose gel. Lipid composition was measured with enzymatic assay kits (Wako Pure Chemicals, Osaka), (for detail see Chapter 2).

Apolipoproteins were obtained from the fractions of fresh human plasma lipoprotein and dissolved in an aqueous solution as described in the Chapter 2.

2. Preparation and modification of [^{3}H]-cholesterol oleate LDL

[$^{1,2-3}$H]-cholesteryl oleate (45.4 Ci/mmol, from Amersham Canada) labeled LDL was prepared according to the method described by Nishisawa, et al and the modifications of labeled LDL were prepared by the method described by Basu et al. (Nishikawa, et al. 1985; Basu, et al. 1976; for detail see chapter 2 and 3). The final specific radioactivity was approximately 10,000 dpm/μg LDL protein. The modified LDL was dialyzed against 12 L PBS for 4 days at 4 ^{0}C. The chemical modifications of LDL were confirmed by electrophoretic mobility in agarose gel.

3. Preparation of microemulsion

Lipid microemulsion was made form egg phosphatidylcholine (from Avanti), and triolein (Sigma) as described (Tajima and Yokoyama, 1983), with an initial weight ratio of triolein/PC 1:1 and a final ratio of 1.25-1, see Chapters 2 and 3. The preparation was stocked at 4 ^{0}C under argon. The microemulsion was used within two weeks (see Chapter 3 for details).

4. Loading cells with radiolabeled cholesterol and labeling of cellular choline-phospholipids

Mouse peritoneal macrophages were obtained from peritoneal lavage as described (Hara, and Yokoyama, 1991). The macrophages were loaded with radiolabeled cholesterol according to the previously described (Hara and Yokoyama 1991) by incubating the cells with the acetylated LDL (labeled at cholesterol ester) in 1 ml 0.2% BSA of RPMI 1640 medium (Flow Laboratories) for 24 h together with [methyl-^{3}H]-choline chloride (Amersham). The cells were then washed 3 times with medium and reincubated without lipoprotein for another 24 h for cholesterol corporation.

Smooth muscle cells were obtained from the entire thoracic aorta of a Sprague-Dawley rat as described previously. Cells were maintained in 10% fetal calf serum medium for 3-4 days until they became subconfluent and the cells were then incubated with radiolabeled cationized LDL (35-40 μg/ml by protein) for about 5 days and [^{3}H]-choline for last 24 h. The labeled cells were washed three times with medium and incubated in the minimum essential medium (MEM) with 0.2% BSA for another 24 h for corporation.

5. Incubation of cells with MCSF, PDGF and phorbol ester

After the appropriate labeling protocol, the cells were preincubated with 100 ng/ml MCSF and 10 ng/ml PDGF (from Sigma) individually or together for 24 h in 1 ml of MEM or RPIM 1640 medium containing 2 mg BSA, as described (Ishibashi, et al., 1990 and Inaba, et al., 1992). The cells were then incubated with 160 nM phorbol myristate acetate (PMA) for 45 min in the same medium (Mendez, et al. 1991). After the incubation, the cells were rinsed three times with medium and then incubated with apoA-I or HDL to examine lipid efflux.

6. Cholesterol efflux from cells

After the treatments, the cells were carefully washed with cell culture medium and then incubated with various concentrations of apoA-I, HDL in 1ml of PRMI 1640 or MEM medium containing 2 mg/ml BSA at 37 °C for various durations. After the incubation, medium was collected and centrifuged at 10,000 rpm for 2 min in a Beckman Microfuge to remove the cell components. The cell layer was washed four times with PBS to remove trace radioactivity (Hara, and Yokoyama, 1991)

7. Analysis of lipid efflux from cells

Lipids were extracted from the medium and cells by chloroform/methanol (2:1) and hexane/isopropyl alcohol (3:2), respectively (Hara and Yokoyama, 1991). The aliquot of the lipid extraction was applied on TLC with a microsyringe, together with standard lipids. After drying, the TLC plates were developed by two steps to isolate cholesterol, cholesterol ester and choline-phospholipids. The developed TLC plates were stained with iodine and each lipid fraction was scraped from the plates for radioactivity counting and for further analysis, see Chapters 2 and 3.

8. Cholesterol oxidation and other methods

Cholesterol loaded SMC were incubated in presence or absence of M-CSF and PDGF plus PMA as described previously. The cells were washed with PBS, chilled to 0 °C and then fixed for 15 min in 1% glutraldehyde. The cells were washed twice with 310 mM sucrose, 0.5 mM NaPi (pH 7.5) solution, and incubated with 3 IU/ml of cholesterol oxidase in the same buffer for indicated time at 37 °C. The cellular lipid was extracted with cold hexane/isopropyl acohol (3:2). The extracts were spotted on TLC and developed in petroleum ether/ethyl ether/acetic acid (80:20:1, v/v)(Lange, Y., 1983, see Chapter 3). The radioactivity in the various spots was determined. Cellular protein was determined by the Lowry's method (Lowry, et al. 1951)

RESULTS AND DISCUSSION

It is reported that both M-CSF and PDGF are produced by foam cells in atherosclerotic lesions. Therefore, they may play a role in regulating cholesterol efflux from foam cells. In order to investigate cellular factors involved in apolipoprotein-mediated cholesterol efflux, SMC were treated with M-CSF, PDGF and PMA. In normal SMC, M-CSF and scavenger receptors are not expressed, while, SMC can respond to PDGF-BB and express M-CSF receptors by coexpression with PDGF receptors (Inaba, 1992). Furthermore, SMC can respond to M-CSF and initiate some macrophage-like biological events including expression of scavenger receptors and phagocytosis. After incubation with PDGF 10 ng/ml and MCSF 100 μg/ml, smooth muscle cells demonstrated uptake of acetylated LDL to become foam cells while untreated smooth muscle cells did not.

1. The effect of M-CSF, PDGF and PMA on cholesterol efflux from SMC

Cholesterol-loaded SMC were preincubated in the presence or absence of 100 ng/ml M-CSF or/and 10 ng/ml PDGF for 24 hours. Cholesterol efflux was determined in the presence of apoAI or HDL. Cholesterol efflux was only slightly enhanced by the treatment of M-CSF or PDGF (Figure IV.1). This suggests that the treatment of cells by M-CSF or PDGF can not increase cholesterol availability in smooth muscle cells.

Cholesterol-loaded SMC were also incubated with 160 nM PMA for 45 min at 37 °C with subsequent A-I-mediated lipid efflux determination. The results showed that 160 nM PMA enhanced cholesterol efflux by approximately 20% (Figure IV.2), suggesting that PKC activation alone is insufficient to largely increase for cholesterol efflux. It was reported that HDL binding with HDL-binding protein on fibroblast membrane activated protein kinase C and translocated cholesterol from the intracellular pool to the plasma membrane, facilitating cholesterol efflux (Mendez, et al., 1991). In our experimental conditions, PMA failed to stimulate cholesterol efflux greatly from SMC. This suggests that the stimulatory effect of PMA on cholesterol efflux may have cell-line specificity.

2. The combined effects of M-CSF, PDGF and PMA on cholesterol efflux from SMC

To further investigate the cellular mechanism of cholesterol efflux, the combined effect of both PMA and growth factors on cholesterol efflux was performed. The cholesterol-loaded SMC were preincubated with 100 ng/ml M-CSF and 10 ng/ml PDGF for 24 hours with additional incubation incorporating 160 nM PMA for the last 45 min. ApoA-I and HDL-mediated cholesterol efflux were then examined. The cholesterol efflux of the treatment group was 2.6-fold higher than that of the control and the cells can take up acetylated LDL. However, the single growth factor and PMA treatment (M-CSF or PDGF plus PMA treatment) enhanced cholesterol efflux only mildly (Figure IV.3 left). M-CSF or/and PDGF treatment alone (without PMA) was insufficient to enhance cholesterol efflux (Figure IV.3 right). This suggests that combined effects (M-CSF+PDGF+PMA) are required to greatly enhance A-I-mediated cholesterol efflux from SMC. Interestingly, the same treatment may only enhance HDL-mediated cholesterol efflux by approximately 30-40%. This increase may be due to the increase of cholesterol efflux mediated by apoA-I which dissociated from HDL. This seems to correlate with the report that HDL-mediated cholesterol efflux is mainly mediated by cholesterol diffusion (Philips, 1987) rather than a cell regulated process. These results may indicate that M-CSF and PDGF plus PMA selectively enhance cholesterol efflux by affecting specific cellular regulatory pathways.

3. Dose- and time-dependence of lipid efflux from SMC after treatment of M-CSF, PDGF and PMA

Cholesterol-loaded and choline-phospholipid-labeled SMC were incubated with M-CSF and PDGF in the presence of PMA as described. Lipid efflux was subsequently determined in indicated concentrations of apoAI. The results demonstrated that cholesterol efflux was enhanced as a function of A-I concentration and saturated at 10 μg/ml of AI without enhancement of PC efflux (Figure IV.4). Since the treatment only increased the cholesterol efflux, not the PC efflux, the ratio of cholesterol/PC in medium was doubled (Figure IV.5).

It appears that the present treatment only affects the specific cellular mechanism involving cholesterol efflux rather than phospholipid efflux. This suggests that enhancement of cholesterol efflux is not mediated through an increasing reactivity of SMC but by changing cholesterol availability for this reaction.

In the time-dependence experiments, 10 μg/ml A-I and 100 μg/ml HDL were used and incubated with the M-CSF, PDGF, and PMA treated cells as described previously. It was shown that no great increase of apoA-I-mediated cholesterol efflux occurred until after 10 hours (Figure IV.6 left). This suggested that a minimum of 10 hours lag was required for cells to respond to the treatment factors. ApoA-I-mediated efflux continually increased during 24 hours, suggesting that the treatment effect still existed up to 24 hours. However, no enhancement of HDL-mediated cholesterol and PC efflux was observed by the same treatment (Figure IV.6 right and Figure IV.7).

Although the mechanism for this cholesterol efflux enhancement is not yet clear, cholesterol accumulation in intracellular pools, such as the lysosome, can lead to poor cholesterol efflux. Therefore, enhancement of cholesterol translocation from intracellular pools to the cell membrane may increase cholesterol efflux. The process of cholesterol translocation may be mediated by protein, vesicles, or other mechanisms (William, et al. 1991). Furthermore, other factors can also affect the cholesterol translocation. In Neimann-Pick C disease, linked to a defect cholesterol transport out of the lysosomes, sphingomyelin accumulation coexists with accumulation of large amounts of free cholesterol (Liscum, et al. 1989). The treatment with 2% dimethyl sulfoxide can reverse the cholesterol accumulation, suggesting that the factors affecting the membrane permeability and lipid solubility may reduce the trapping of cholesterol in lysosomes as well (Mackie, et al. 1989). It remains to be identified whether or not the increased cholesterol efflux by the present treatment is mediated through enhancement of cholesterol translocation from intracellular pools to the plasma membrane. Therefore, independent and direct measurement of the plasma membrane cholesterol pool may help to address this issue.

On the other hand, cholesterol efflux can also be enhanced without change of cholesterol mass in cell membrane. As reported, cholesterol may distribute among different membrane cholesterol-domains (Rothblat, et al. 1992). The rate of cholesterol efflux from the different domains is different according to the strength of lipid interaction within each domain. The packing forces between lipid molecules is weaker in lipid-poor domains than that in lipid-rich domains, therefore, cholesterol molecules desorb more easely from the lipid-poor membrane surface. Based on the cholesterol distribution among the domains, cholesterol efflux is assigned to the fast, intermediate and slow domains. SMC possess only the slow efflux domain (Mahlberg, et al., 1992), suggesting that cholesterol efflux tends to be slower from SMC. This indicates that the factors changing cholesterol distribution on the membrane may alter the rate of cholesterol efflux without enlarging the cholesterol mass in the membrane. Additionally, the ratio of sphingomyelin/cholesterol influences cholesterol efflux as well. Cholesterol efflux is slower from sphingomyeline-rich membranes than that from sphingomyelin-poor membranes (Gold, et al. 1990). Therefore, the distribution or content of sphingomyelin in the membranes may affect cholesterol efflux as well. It remains to be identified by which mechanism the present treatment enhances the cholesterol efflux from SMC.

4. Effect of M-CSF and PDGF plus PMA on cholesterol efflux from macrophages

To compare the effect of M-CSF, PDGF and PMA on cholesterol efflux from macrophages, cholesterol-loaded macrophages were treated by M-CSF, PDGF and PMA as described. ApoA-I-mediated cholesterol efflux was enhanced only about 30-40% and no significant increase was observed in HDL-mediated lipid efflux by the treatment (Figure IV.8). This suggests that the enhancement of cholesterol efflux from SMC is cell-line specific. Normally, free-apolipoproteins can remove up to 50% cellular cholesterol from macrophage. This implies that cholesterol efflux processes in mouse macrophages are already very active in the untreated cells, therefore, the treatment may not able to increase the overall function of cholesterol efflux further from macrophages.

5. Other relative results

Protein content was determined to examine cell growth after the treatment. The difference of protein concentration between the control and treated groups was not significant. This suggests that the enhancement of A-I-mediated cholesterol efflux is independent of the cell protein concentration.

Cholesterol oxidase was utilized to determine membrane surface cholesterol (Figure IV.9). The cholesterol oxidase method was unable to demonstrate significant changes before and after the treatment. This may suggest that the enhancement of cholesterol efflux is not related to the increase of cholesterol mass on cell membrane. On the other hand, the cholesterol oxidase may be unable to indicate the real cholesterol availibility in the cells because it cannot reflect the cholesterol exchange rate between the cellular cholesterol pools. Total PKC activity in cell homogenization had no significant detectable change in our experimental conditions between the treated and untreated SMC (data not show).

However, more efficient analysis of membrane cholesterol and other cellular function, for instant, hydrolysis of storeed cholesterol ester, esterification of free cholesterol, and translocation of intracellular cholesterol are required to understand the mechanism.

REFERENCES

Aviram, M., Bierman, E. L. and Oram, J. F. High density lipoprotein stimulates sterol translocation between intracellular and plasma membrane pools in human monocyte-derived macrophages. J. lipid Res. 1989; 30: 65-76.

Basu, S. K., Goldstein, J. L., Anderson, R. G. W. and Brown, M. S. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial fibroblasts. Proc. Natl. Acad. Sci. U. S. A. 1976; 73: 3178-3182.

Castro, G. R. and Fielding, C. J. Early incorporation of cell-derived cholesterol into preb-migrating high density lipoprotein. Biochemistry. 1988; 27: 25-29.

Gary, L. M., Reasoner, J., Gilbert, T. and Sundquist, K. O. Cloning and expression of a cellular high density lipoprotein-binding protein that is up-regulated by cholesterol loading of cells. J. Biol. Chem. 1992; 267: 12131-12141..

Gold, J. C. and Phillips, M. C. Effect of membrane lipid composition on the kinetics of cholesterol exchange between lipoproteins and different species of red blood cells. Biochim. Biophy. Acta. 1990; 1027: 85-92.

Gordon, T., Castelli, W. P., Hjortland, M. C. and Kannel, W. B. High density lipoprotein as a protective factor against coronary heart disease: Am. J. Med. 1977; 62: 707-714.

Hara, H. and Yokoyama, S. Interaction of free apolipoproteins with macrophages. J. Biol. Chem. 1991; 266: 3080-3086.

Graham, D. L. and Oram, J. F. Identification and characterization of a high density lipoprotein-binding protein in cell membranes by ligand blotting. J. Biol. Chem. 1987; 262: 7439-7442.

Inaba, T., Yamada, N., Gotoda, T. and Shimano, H., et al. Expression of M-CSF receptor encoded by c-fms on smooth muscle cell derived from arteriosclerotic lesion. J. Biol. Chem. 1992; 267: 5693-5699.

Inaba,T., Gotoda, T., Shimano, H and Shimano, M, et al. Platelet-derived growth factor induces c-fms and scavenger receptor genes in vascular smooth muscle cells. J. Biol. Chem. 1992; 267: 13107-13112.

Inoue, I., Inaba, T., Motoyoshi, K. and Harada, K. Macrophage colony stimulating factor prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits. Atherosclerosis. 1992; 93: 245-254.

Ishibashi, S., Inaba, T., Shimano, H., and Harada, K., et al. Monocyte Colony-stimulating factor enhances uptake and degradation of acetylated low density lipoprotein and cholesterol esterification in human monocyte-derived macrophages. J. Biol. Chem. 1990; 265: 14109-14117.

Johnson, J. W., Mahlberg, F. H., Rothblat, G. H. and Philips, M. C. Cholesterol transport between cells and high-density lipoproteins. Bioch. Biophy. Acta. 1991; 1985: 273-298.

Komaba, A., Li, Q., Hara, H. and Yokoyama, S. Resistance of smooth muscle cells to assembly of high density lipoproteins with extracellular free apolipoprotein and to reduction of intracellularly accumulated cholesterol. J. Biol. Chem. 1992; 267: 17560-17566.

Lange, Y. and Ramos, B. V. Analysis of the distribution of cholesterol in the intact cells. J. Biol. Chem. 1983; 258: 15130-15134.

Li, Q., Komaba, A. and Yokoyama, S. Cholesterol is poorly available for free apolipoprotein-mediated cellular lipid efflux from smooth muscle cells. Biochemistry. 1993; 32: 4597-4603.

Liscum, L., Ruggiero, R. M. and Faust, J. R. The intracellular transport of low density lipoprotein-derived cholesterol is defective in Niemann-Pick type C fibroblasts. J. Cell Biol. 1989; 108: 1625-1636.

Lowry, O. H, Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the folin phenol reagent. J. Biol. Chem. 1951; 193: 265-275.

Mackie, E. J., Dwyer, N. K., Vanier, M. T. and Sokol, J. et al. Type C Niemann-Pick disease: dimethyl sulfoxide moderates abnormal

LDL-cholesterol processing in mutant fibroblasts. Biochim. Biophy. Acta. 1989; 1006: 219-226.

Mahlberg, F. H. and Rothblat, G. H. Cellular cholesterol efflux. J. Biol. Chem. 1992; 267: 4541-4550.

McKnight, G. L. Reasoner, J., Gilbert, T. and Sundquist, K. O., et al. Cloning and expression of a cellular high density lipoprotein-binding protein that is up-regulated by cholesterol loading of cells. J. Biol. Chem. 1992; 267: 12131-12141.

Mendez A J., Oram J F. and Bierman E L. Protein Kinase C as a mediator of high density lipoprotein receptor-dependent efflux of intracellular cholesterol. J. Biol. Chem. 1991; 266: 10104-10111.

Miida, T., Fielding, C. J. and fielding, P. E. Mechanism of transfer of LDL-derived free cholesterol to HDL subfraction in human plasma. Biochemistry. 1990; 29: 10469-10474.

Nishikawa, O. and Yokoyama, S. et al. A method for direct incorporation of radiolabeled cholesterol ester into human plasma low-density-lipoprotein: preparation of trace substrate for cholesterol ester transfer reaction between lipoproteins. J. Biol. (Tokyo) 1985; 99:295-301.

Oram, J. F., Mendez, A. J., Slotte, J. P. and Johnson, T. F. High density lipoprotein apolipoproteins mediate removal of sterol from intracellular pools but not from plasma membranes of cholesterol-loaded fibroblasts. Arterioscl. and Throm. 1991; 11: 403-414.

Phillips, M. C., Johnson, W. J. and Rothblat, G. H. Mechanisms and consequences of cellular cholesterol exchange and transfer. Bioch. Biophy. Acata. 1987; 906: 233-276.

Rothblat, G. H., Mahlberg, F. H., Johnson, W. J. and Phillips, M. C. Apolipoproteins, membrane cholesterol domains, and the regulation of cholesterol efflux. J. Lipid Res. 1992; 33: 1091-1097

Shimano, H., Yamada, N., Ishibashi, S. and Harada, K., et al. Human monocyte-stimulating factor enhances the clearance of lipoproteins containing apolipoprotein B-100 via both low density lipoprotein receptor-dependent and -independent pathways in rabbits. J. Biol. Chem. 1990; 265: 12869-12875.

Slotte, J. P., Oram, J. F. and Bierman, E. L. Binding of high density lipoprotein to cell receptors promotes translocation of cholesterol from intracellular membranes to the cell surface. J. Biol. Chem. 1987; 262: 12904-12907.

Tajima, S. and Yokoyama, S. J. Effect of lipid size on association of apolipoprotein. Biol. Chem. 1983; 258: 10073-10082.

William, J. J., Mahlberg, F. H., Rothblat, G. H. and Phillips, M. C. Cholesterol transport between cells and high-density lipoproteins. Bioch. Biophy. Acta. 1991; 1085: 273-298.

Figure IV.1. The effect of MCSF and PDGF on cholesterol efflux from SMC. SMC were loaded with cholesterol by incubating with cationized LDL (as described in experiment procedure). The cells were preincubated for 24 hours in the presence and absence of 100 ng/ml MCSF and 10 ng/ml of PDGF in 1 ml of 0.2% BSA MEM medium. The cells were washed 4 times and then incubated with 10, 20 μg/ml apoA-I or 100 μg/ml HDL for another 24 hours. After the incubation, lipids in the medium and cells were extracted, separated and counted. The background was subtracted from each experimental group. The cholesterol efflux is presented as CPM/per mg cellular protein.

MCSF: monocyte colony-stimulating factor;
PDGF: platelet-deriveded growth factor;
PMA: phorbol myristate acetate;
SMC: smooth muscle cells, Mp: macrophages

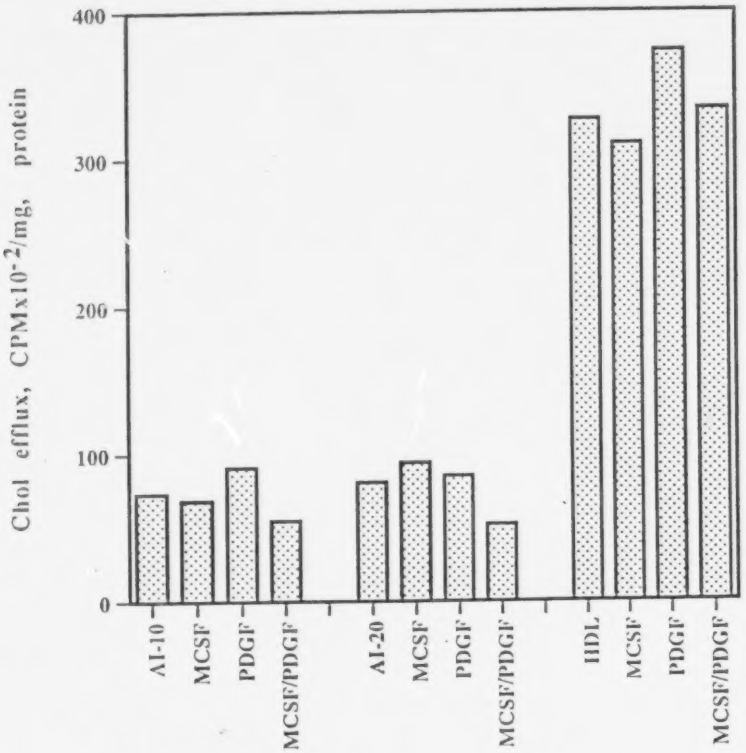

Chol efflux, CPMx10-2/mg, protein
400
300
200
100
0
AI-10
MCSF
PDGF
MCSF/PDGF
AI-20
MCSF
PDGF
MCSF/PDGF
HDL
MCSF
PDGF
MCSF/PDGF

Figure IV.1

Figure IV.2. The effect of PMA on cholesterol efflux from SMC. Cholesterol-loaded SMC were preincubated with 160 nM PMA for 45 min at 37 ^{0}C in 1 ml of MEM medium containing 0.2 % BSA. Then the cells were washed and incubated with 5 µg/ml apoA-I for 24 hours. The medium was collected and the lipids were extracted as described before. The lipid efflux was presented as CPM/per mg cellular protein.

PMA: phorbol myristate acetate;

SMC: smooth muscle cells, Mp: macrophages

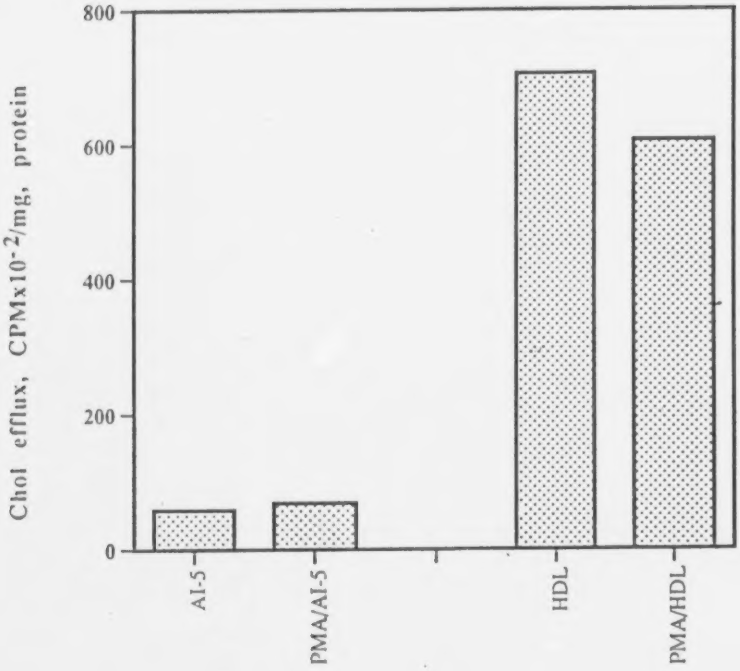
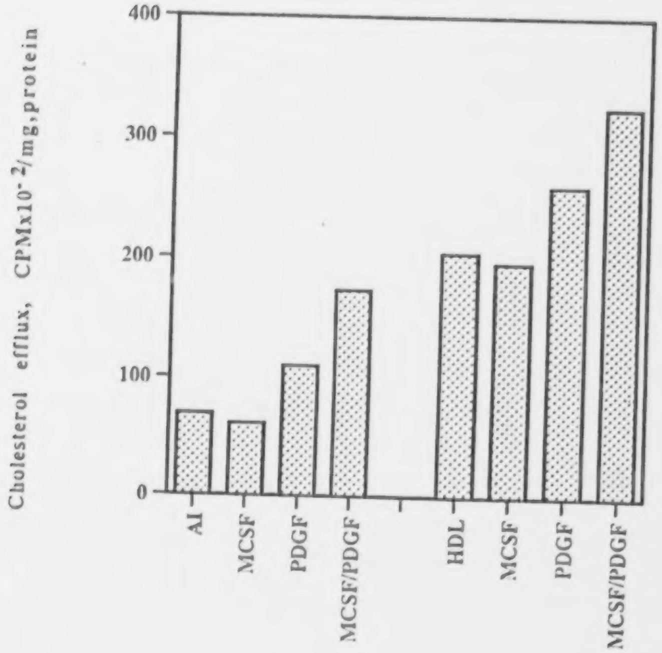

Chol efflux, CPMx10-2/mg, protein
800
600
400
200
0
AI-5
PMA/AI-5
HDL
PMA/HDL

Figure IV.2

Figure IV.3. Effect of MCSF, PDGF and PMA on cholesterol efflux from SMC. SMC were loaded with cholesterol by incubating with cationized LDL (as described in experiment procedure). Cholesterol-loaded SMC were preincubated for 24 hours in the presence and absence of 100 ng/ml MCSF and 10 ng/ml of PDGF in 1 ml of 0.2% BSA MEM medium. For the last 45 min, PMA was added and incubated together with the same medium. The cells were washed 4 times with medium and then incubated with 10 µg/ml apoA-I or 100 µg/ml HDL for another 24 hours. After the incubation, lipid in the medium and in the cells were extracted, separated by TLC. Radioactivity in PC and cholesterol fractions was counted. The background were subtracted from each value. The cholesterol efflux were presented as CPM/per mg cellular protein.

MCSF: monocyte colony-stimulating factor;
PDGF: platelet-deriveded growth factor;
PMA: phorbol myristate acetate;
SMC: smooth muscle cells, Mp: macrophages



PMA (+)

Figure IV.3

Figure IV.4. Effect of MCSF, PDGF and PMA treatment on lipid efflux as the function of apoA-I concentrations. SMC were loaded with cholesterol by incubating with cationized LDL (as described in experiment procedure). The SMC were incubated for 24 hours in present and absent of 100 ng/ml MCSF and 10 ng/ml PDGF in 1 ml of 0.2% BSA MEM medium. For the last 45 min, 160 nM PMA was added and incubated together with previous treatment factors. The cells were washed 4 times and then incubated with apoA-I as indicated concentrations or with 100 μg/ml HDL. At the end of incubation, lipids were extracted from medium and cells as described in the experiment procedure. The background of cholesterol efflux in the presence of only 0.2% BSA was subtracted from each value. The lipid efflux is presented as CPM/per mg cellular protein.

MCSF: monocyte colony-stimulating factor;
PDGF: platelet-deriveded growth factor;
PMA: phorbol myristate acetate;
SMC: smooth muscle cells, Mp: macrophages

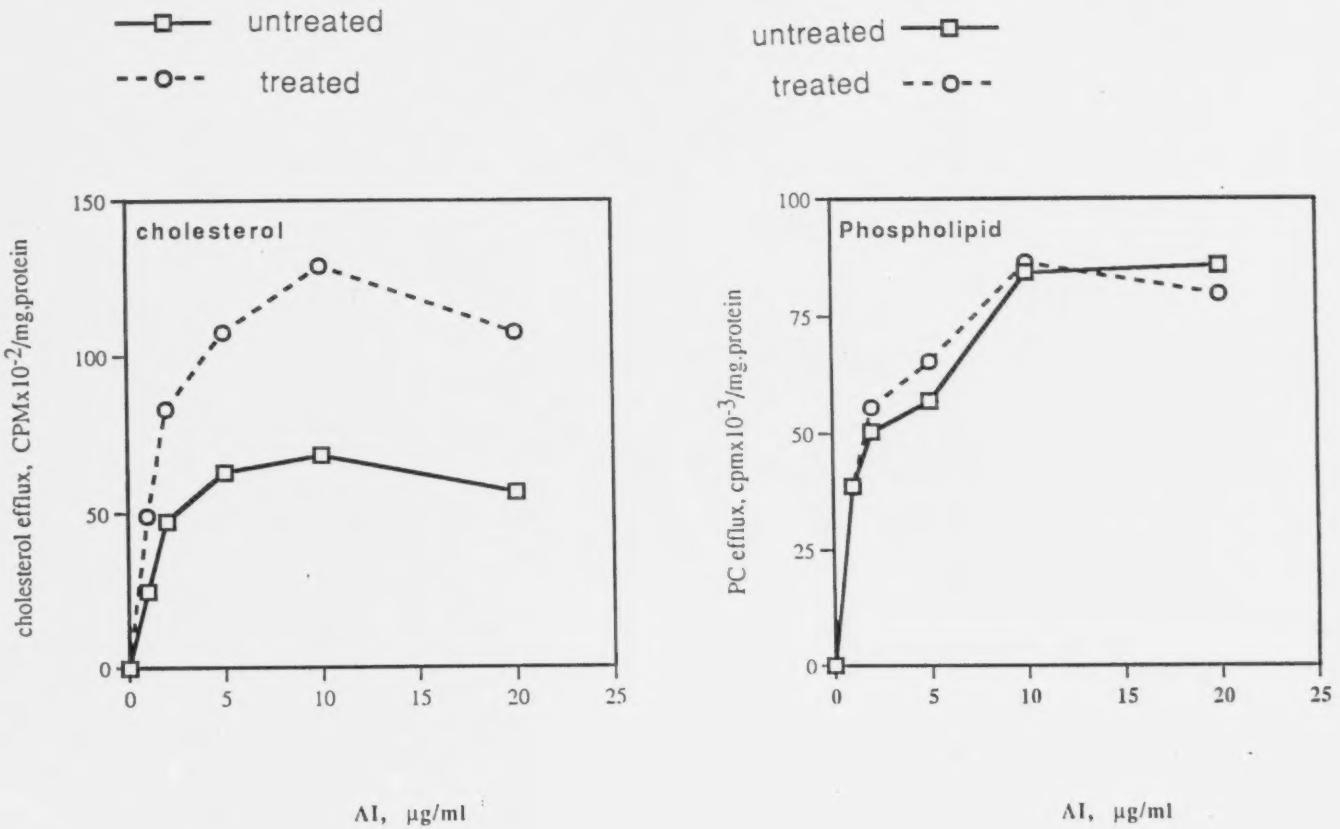

untreated
treated
cholesterol
cholesterol efflux, CPMx10^{-2}/mg.protein
150
100
50
0
0
5
10
15
20
25
AI, µg/ml
untreated
treated
Phospholipid
PC efflux, cpmx10^{-3}/mg.protein
100
75
50
25
0
0
5
10
15
20
25
AI, µg/ml

Figure IV.4

Figure IV.5. The enhancement of cholesterol efflux after the growth factors and PMA treatment (the ratio of cholesterol/PC). The cells were treated as described in Fig. 4. The panel is the ratio of cholesterol/PC of efflux medium in different apoA-I concentrations.

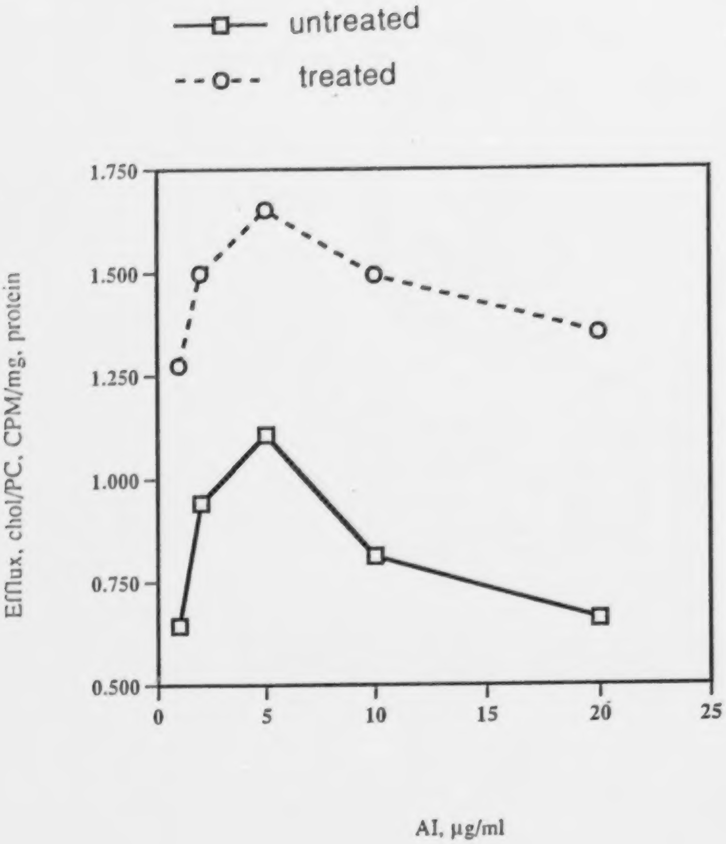

untreated
treated
Efflux, chol/PC, CPM/mg, protein
1.750
1.500
1.250
1.000
0.750
0.500
0
5
10
15
20
25
AI, µg/ml

Figure IV.5

Figures IV.6 and 7. Effect of MCSF, PDGF and PMA treatment on lipid efflux as the function of times. SMC were loaded with cholesterol by incubating with cationized LDL (as described in experiment procedure). Cholesterol-loaded SMC were incubated for 24 hours in present and absent of 100 ng/ml MCSF and 10 ng/ml of PDGF in 1 ml of 0.2% BSA MEM medium. For the last 45 min incubation, PMA was added and incubated together with previous treatment factors. Cells were washed 4 times and then incubated with 10 ug/ml of apoA-I or 100 ug/ml of HDL for indicated times. After the incubation, lipids in the medium and cells were extracted, separated and counted. The background of cholesterol efflux in the presence of only 0.2% BSA was subtracted from each value. The lipid efflux is presented as CPM/ per mg cellular protein.

MCSF: monocyte colony-stimulating factor;
PDGF: platelet-deriveded growth factor;
PMA: phorbol myristate acetate;
SMC: smooth muscle cells, Mp: macrophages

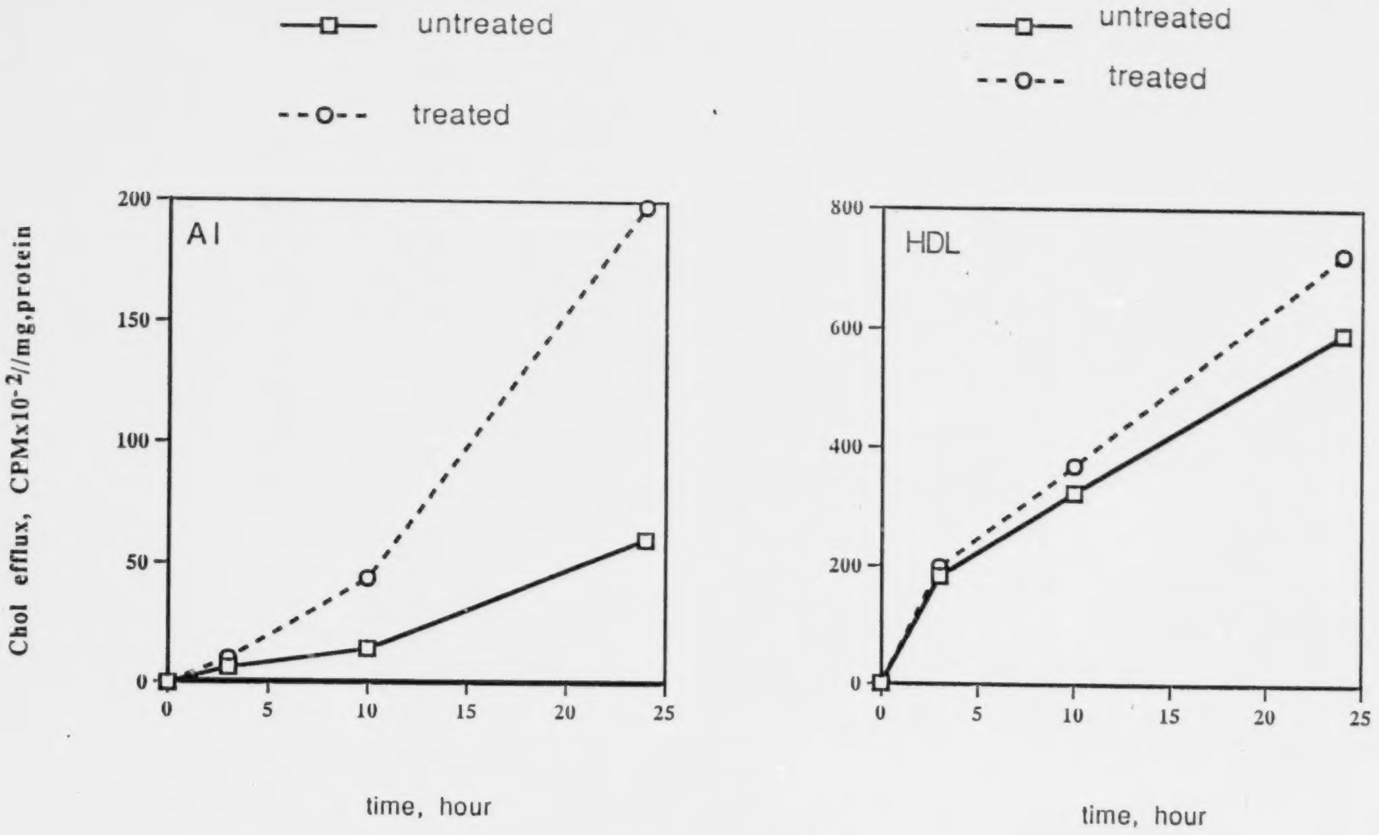

untreated
treated
untreated
treated
Chol efflux, CPMx10-2//mg,protein
AI
HDL
200
150
100
50
0
800
600
400
200
0
0
5
10
15
20
25
time, hour
time, hour

Figure IV.6

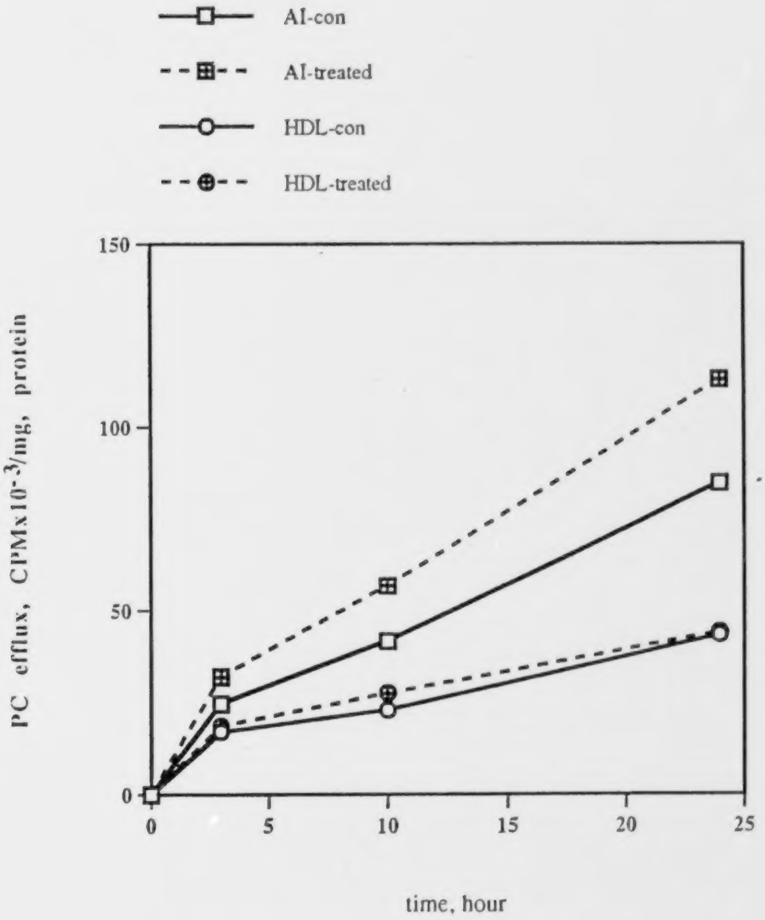

AI-con
AI-treated
HDL-con
HDL-treated
PC efflux, CPMx10^{-3}/mg, protein
0
50
100
150
0
5
10
15
20
25
time, hour

Figure IV.7

Figure IV.8. Effect of MCSF, PDGF and PMA on cholesterol efflux from Macrophages. Cholesterol-loaded macrophages were incubated for 24 hours in present and absent of 120 ng/ml MCSF and 10 ng/ml of PDGF in 1 ml of 0.2% BSA MEM medium. At the last 45 min of incubation, PMA was added and incubated together with growth factors. Cells were washed 4 times with medium to remove the treatment factors and then incubated with 10 µg/ml apoA-I or 100 µg/ml HDL for another 24 hours. After the incubation, cholesterol in the medium and in the cells were extracted and measured. The background of cholesterol efflux in the presence of only 0.2% BSA were subtracted from each value. The cholesterol efflux was presented as CPM /
per mg cellular protein.

MCSF: monocyte colony-stimulating factor;
PDGF: platelet-deriveded growth factor;
PMA: phorbol myristate acetate;
SMC: smooth muscle cells, Mp: macrophages

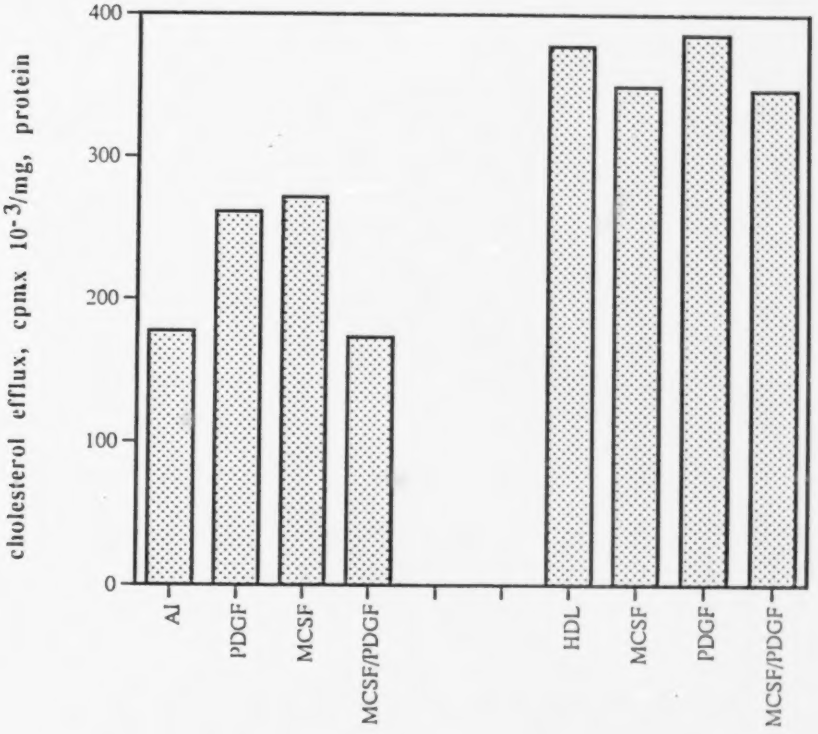

cholesterol efflux, cpmx 10^{-3}/mg, protein
400
300
200
100
0
AI
PDGF
MCSF
MCSF/PDGF
HDL
MCSF
PDGF
MCSF/PDGF
Treatment

Figure IV.8

Figure IV.9. Oxidation of cellular cholesterol. The cholesterol-loaded SMC were incubated with M-CSF, PDGF and PMA as described. The cells were treated with 3 IU/ml cholesterol oxidase for indicated times as described in the "method". After the treatment, the cellular lipids were extracted and separated. The data presents the ratio of cholestone /unesterified cholesterol as the function of the treatment time. The background of oxidase untreated dish was subtracted from each data.

MCSF: monocyte colony-stimulating factor;
PDGF: platelet-deriveded growth factor;
PMA: phorbol myristate acetate;
SMC: smooth muscle cells, Mp: macrophages

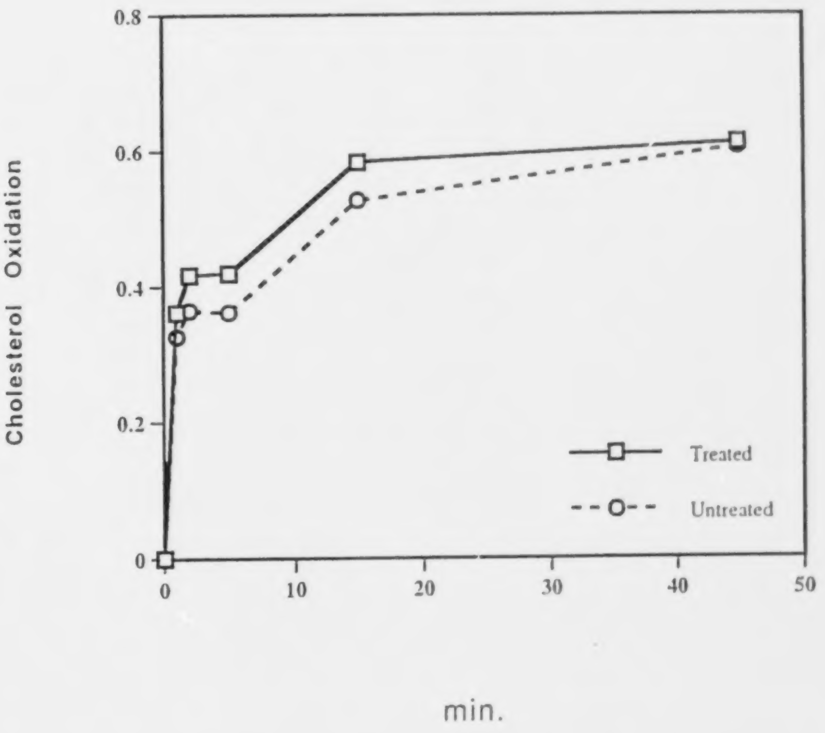

0.8
0.6
0.4
0.2
0
Cholesterol Oxidation
0
10
20
30
40
50
min.
Treated
Untreated

Figure IV.9

Chapter V.

Effect of Proteolysis on Lipid Efflux from Smooth Muscle Cells and Macrophages

INTRODUCTION

The process of cholesterol efflux seems to involve not only physiochemical factors, such as membrane and acceptor lipid components, but also involves some cellular regulatory mechanism such as surface binding protein. HDL binding protein(s) has been demonstrated by ligand blotting experiments in fibroblasts, endothelial and smooth muscle cells, hepatocytes, and macrophages (Biesbroeck, et al. 1983; Havekes, et al. 1984; Hoeg, et al. 1985; Schmitz, et al. 1985). One of them has been cloned and sequenced (Graham, et al. 1987; Tosuka, et al. 1987; Mcknight, et al. 1992). Yet, the studies on the function of these binding proteins are controversial. It has been found that the activity of these proteins is up-regulated in presence of cholesterol and reversed when cells are exposed to cholesterol-free medium (Oram, et al. 1983). More recently, it is demonstrated that the binding of HDL with the binding protein stimulates the translocation of excess intracellular sterol to the cell surface where it is accessible for removal by HDL. This suggests that the functions of these binding proteins are related to the removal of excess intracellular sterol from cells (Oram, et al., 1990; 1991). But the mechanism of the binding protein-mediated cholesterol efflux is not fully understood. On the other hand, it is also evidenced that HDL-mediated cholesterol efflux is a nonspecific exchange process. In this process, cholesterol molecules on cell membranes diffuse down the cholesterol gradient between the cell membrane and the HDL particle until collide HDL particles (William, et al. 1991). Therefore it is controversial whether cholesterol efflux is a nonspecific diffusion process or a protein-mediated specific process.

It was found in our lab that lipid-free apolipoproteins can mediate net cholesterol efflux (Hara, et al. 1991). These apolipoproteins are able to remove cholesterol from both cell membranes and intracellular pools (Mahlberg. 1991), but the mechanism remains unclear. In our previous experiment, it was found that SMC were very resistant to apolipoprotein-mediated cholesterol efflux but not to HDL-mediated cholesterol efflux. This indicates that the mechanism of apoA-I and HDL-mediated cholesterol efflux may be different. In contrast to that of SMC, apolipoprotein can remove about 30-50% cellular cholesterol from macrophages, suggesting the pathway of apolipoprotein-mediated cholesterol efflux is very active in macrophages. These two distinct cholesterol efflux pathways would become good tools to study the mechanism of cholesterol efflux.

We attempted to modify cell surface proteins with trypsin and measure lipid efflux from both smooth muscle cells and macrophages. Results showed that apoAI-mediated cholesterol efflux from macrophages and smooth muscle cells was totally suppressed by trypsin treatment. On the other hand, HDL-mediated cholesterol efflux was only partially inhibited. This implied that cholesterol efflux mediated by apoAI requires cell surface integrity, while HDL-mediated cholesterol efflux may be more dependent on the nonspecific diffusion process which was not sensitive to trypsin treatment.

MATERIALS AND METHODS

Macrophages and smooth muscle cells were loaded with acetylated LDL 50 µg/ml for 24 hours and cationized LDL 40 µg/ml for 5 days, respectively. The preparations of modified LDL, HDL and apolipoproteins were the same as described before.

1. Trypsin treatment

The cholesterol-loaded cells were chilled on ice and washed with cold PBS. The cells were incubated with 4 mg/ml trypsin at 4 °C for indicated times. After trypsin treatment, the cells were washed twice with cold PBS containing 2 mg/ml trypsin inhibitor (from Sigma) and twice with PBS. This procedure of trypsin treatment has been shown to remove intact [^{125}I]-HDL$_3$ which previously bound to cell surface (Oram, et al. 1987).

2. Cholesterol efflux

The trypsin treated cells were incubated with apoAI, HDL and microemulsion for 24 hours at 37 °C as described previously. At the end of incubation, the medium was collected and centrifuged 10,000 rpm at 4 °C to remove cell residue. Lipids in the medium and cells were extracted, separated and measured as described before.

3. Other method

Protein was determined by Lowry's method and cell viability was examined using 1% trypan blue.

RESULTS AND DISCUSSIONS

Previous data from our laboratory have demonstrated that apolipoproteins can remove as much as 30%-50% cellular cholesterol from cholesterol-loaded macrophages, while the efflux from SMC was only 2-5% of cellular cholesterol. However, the HDL-mediated cholesterol efflux from both cells was similar. To understand this difference, macrophages were treated with 0.4% trypsin for 10 min at 4 °C. After the treatment, the cells were carefully washed 4 times with 2 mg/ml trypsin inhibitor and PBS. This intensive washing can totally block the function of trypsin (Oram, et al. 1987). The treated and untreated cells were incubated with apoAI, HDL and microemulsion for 3 hours or 24 hours to determine lipid efflux. Results showed that 0.4% trypsin treatment almost completely inhibited apoAI-mediated cholesterol efflux from macrophages in both 3 and 24 hours incubation groups (Figure V.1. A), while the treatment only reduced HDL and microemulsion-mediated cholesterol efflux by 35% and 29% respectively (Figure V.1.A). Cell viability was over 95% and cell shape did not change at the end of treatment (data not shown). This indicated the changes in cholesterol efflux were not caused by the change of cell viability. This suggested that apoA-I-mediated cholesterol efflux from macrophages requires the membrane integrity. The membrane protein components may play a major role in apolipoprotein-mediated cholesterol efflux. It was assumed that trypsin treatment modified the surface protein and suppressed the interaction between apoA-I and the cell membrane. This led to the total inhibition of apoA-I-mediated cholesterol efflux. The time course of the lipid efflux showed that the effect of trypsin treatment on cholesterol efflux lasts even after 24 hours. This suggests that the recovery of the surface protein involved in cholesterol efflux may require more than 24 hours. However, trypsin treatment only partially inhibited HDL-mediated cholesterol efflux. It has been proposed that the binding of HDL with cell membrane can only induce cholesterol mobilization from intracellular cholesterol pool to membrane pool, while cholesterol efflux direct from membrane pool does not require this interaction. Thus, trypsin treatment may only inhibit cholesterol movement from intracellular pool to the surface if the cholesterol translocation process is induced by HDL surface binding. This may be responsible for the partially reduced cholesterol efflux mediated by HDL. However, the reduced microemulsion-mediated cholesterol efflux should only reflect a nonspecific effect of trypsin treatment on cell membranes and this effect was the same as the effect on the HDL-mediated cholesterol efflux. The data also showed that 0.4% trypsin inhibited >90% apoA-I-mediated PC efflux but inhibited HDL and microemulsion-mediated PC efflux by only 50% (Figure V.1. B).

To investigate the role of surface protein in lipid efflux from SMC, cholesterol-loaded SMC were incubated with 0.4% trypsin for indicated times at 4 °C. The cells were washed and lipid efflux was measured after the cells were incubated 24 h with apoAI or HDL. The time course of trypsin treatment showed that the short time of trypsin treatment (2-10 min) did not reduce the lipid efflux. However, the prolonged treatment completely inhibited apoA-I-mediated cholesterol efflux. On the other hand, the prolonged trypsin treatment only partially reduced HDL-mediated lipid efflux. This suggested that the trypsin-sensitive membrane component was required for apolipoprotein-mediated cholesterol efflux from SMC, while this component did not play such an important role in HDL-mediated lipid efflux. This is consistent with the observation in macrophages and demonstrates again that the mechanism of apolipoprotein-mediated and HDL-mediated cholesterol efflux are different from that of HDL. It has been assumed that free apolipoproteins (mainly apoA-I) dissociated from HDL surface interact with cell membranes and mediate lipid efflux by generating HDL-like particles. These particles, composed of cellular lipids, can contribute to the overall HDL-mediated lipid efflux. In this way, when the apolipoprotein-mediated lipid efflux is suppressed by trypsin treatment, the overall HDL-mediated lipid efflux may be reduced as well.

Thus the trypsin treatment totally inhibited apoAI-mediated cholesterol efflux from macrophages and SMC, suggesting that interaction of the apolipoprotein with cell membrane may play an important role in cholesterol efflux from macrophages and SMC. But it is not the case in HDL-mediated cholesterol efflux. Cholesterol efflux was only partially inhibited after trypsin treatment.

Previous data showed that apoA-I-mediated PC efflux is very similar in both macrophages and SMC although cholesterol efflux from macrophages is much faster than that from SMC (Chapter 3). The weight ratio of PC to cholesterol in lipid efflux from SMC was 2-6, while in macrophages is only about 0.4. This indicated that PC efflux from SMC is much faster than that of cholesterol. There are several possibilities which may lead to this lipid efflux: 1) differences in lipid composition of macrophage and smooth muscle cell membranes, which may directly affect the efflux by supplying different amounts of the lipid available for the efflux; 2) different factors involved in lipid efflux from both cells, such as protein-protein or lipid-lipid and protein-lipid interactions. To examine the role of surface protein in phospholipid efflux from SMC, PC efflux was measured after trypsin treatment (Figure V.2). The data showed that apoA-I-mediated PC efflux was inhibited after 15 min with trypsin treatment (Figure V.2. left). HDL-mediated PC efflux was reduced by about 50% (Figure V.2. right). Thus, the effect of trypsin treatment on the PC efflux was basically the same as that on cholesterol efflux. Kawano et al., (1993) proposed that transfer of cellular lipids to plasma is made up of two separate mechanisms: specific and nonspecific pathways. The desorption rate of PC is much slower than that of cholesterol. As a result, the efflux of PC through the nonspecific mechanism which is dependent on the physical desorption of PC is much slower. This has been demonstrated in previous experiments that protein-free microemulsion mediated a faster cholesterol efflux and a slower PC efflux (Chapter 2). Therefore, it is assumed that the faster PC efflux mediated by apoA-I is due to a specific mechanism compared to the nonspecific efflux mediated by microemulsion. This specific process may involve a membrane component which interacts with apolipoprotein and increases the rate of PC desorption. Thus the inhibition of PC efflux by the trypsin treatment may attribute to modification of the surface protein which may be required for specific PC efflux pathway. However, for further conclusion of the mechanism, it is necessary to determine the function of the surface protein by a more accurate method such as using a specific antibody against the binding protein.

REFERENCES

Biesbroeck, R., Oram, J. F., Albers, J. F., and Bierman, E. L. Specific high-affinity binding of high density lipoprotein to culture human skin fibroblasts and arterial smooth muscle cells. J. Clin. Invest. 1983; 71: 525-39.

Graham, D. L., and Oram, J. F. Identification and characterization of a high density lipoprotein-binding protein in cell membrane by ligand blotting. J. Biol. Chem.1987; 262: 7439-7442.

Havekes, L., Schouten, D., Hinsbergh, V. V., and Wit, E. Characterization of the binding of apolipoprotein E-free high density lipoprotein to culture human endothelial cells. Biochem. Biophys. Res. Commun. 1984; 122 ; 785-790.

Hoeg, J. M., Demmosty, S. J., Stenphen, J., and Gregg, R. E., et al. Characterization of a human hepatic receptor for high density lipoprotein. Arteriosclerosis. 1985; 5: 228-237.

Kawano, M., Miida, T., Fielding, C. J., and Fielding, P. E. Quantitation of preb-HDL-dependent and nonspecific components of the total efflux of cellular cholesterol and phospholipid. Biochemistry. 1993; 32: 5025-5028.

Mahlberg, F. H., and Glick, J. M., Lund-Katz, S. and Rothblat, G. H. Influence of apolipoproteins AI, AII, and Cs on the metabolism of membrane and lysosomal cholesterol in macrophages. J. Biol. Chem. 1991; 266: 19930-19937.

McKnight, G. L., Reasoner, J., Gilbert, T., and Sundquist, K. O. Cloning and expression of a cellular high density lipoprotein-binding protein that is up-regulated by cholesterol loading of cells. J. Biol. Chem. 1992; 267: 12131-12141.

Oram, J. F., Brinton, E. A., and Bierman, E. L. Regulation of high density lipoprotein receptor activity in cultured human skin fibroblasts and human arterial smooth muscle cells. J. Clin. Invest. 1983; 72: 1611-1621.

Oram, J. F., Johnson, C. J., and Brown, T. A. Interaction of high density lipoprotein with its receptor on cultured fibroblasts and macrophages. J. Biol. Chem. 1987; 262: 2405-2410.

Oram, J. F., Mendez, A. J., Slotte, J. P., and Johnson, T. F. High density lipoproteins mediate removal of sterol from intracellular pools but not from plasma membranes of cholesterol-loaded fibroblasts. Arterioscl. and Throm. 1990; 11: 403-414

Tosuka, M. and Fidge, N. Purification and characterization of two high-density-lipoprotein-binding proteins from rat and human liver. Biochem. J. 1989; 261: 239-244.

Figure V.1. The effect of trypsin treatment on lipid efflux from macrophages. Mouse peritoneal macrophages were loaded with radiolabeled cholesterol and choline phospholipids as described before. The cells were treated by trypsin for 10 min at 4 ^{0}C then incubated with apoAI (2 μg/ml and 5 μg/ml), human HDL (100 μg/ml) or microemulsion to examine the lipid efflux. The background with 0.2% BSA was subtracted from the data. Panel A: cholesterol efflux; B: phospholipid efflux.

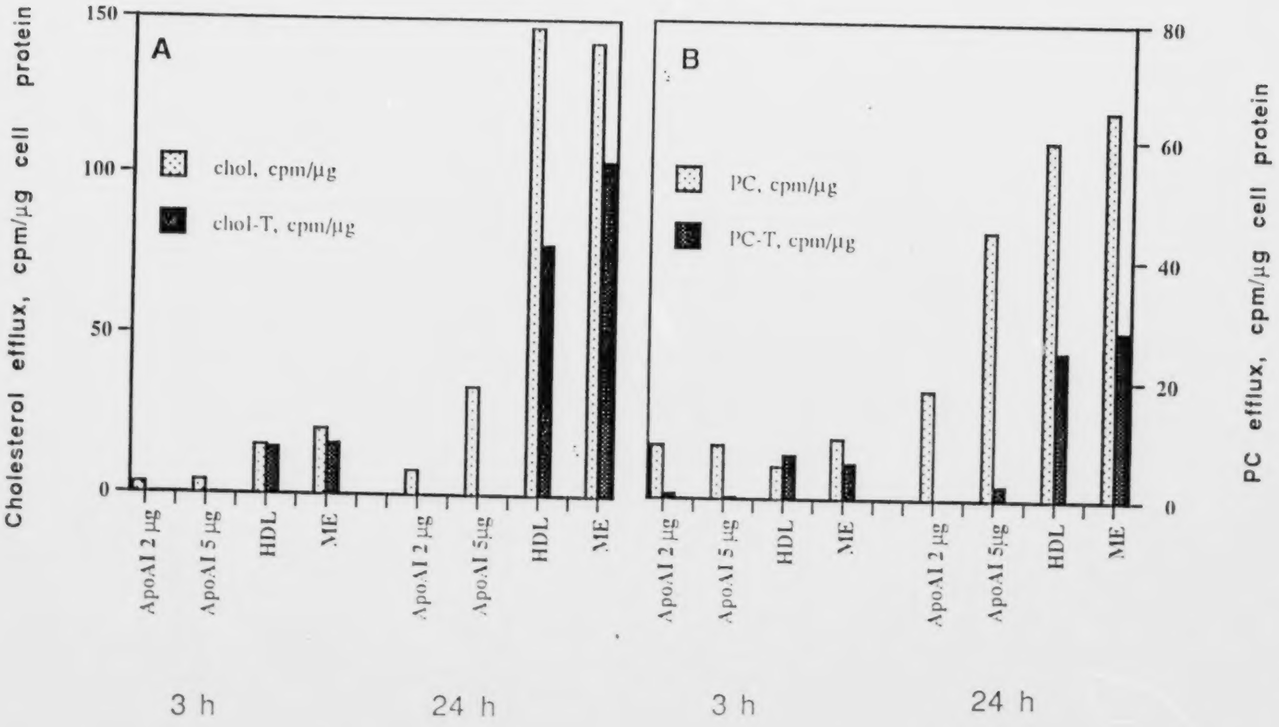

A
chol, cpm/µg
chol-T, cpm/µg
Cholesterol efflux, cpm/µg cell protein
150
100
50
0
ApoAI 2 µg
ApoAI 5 µg
HDL
ME
ApoAI 2 µg
ApoAI 5µg
HDL
ME
3 h
24 h
B
PC, cpm/µg
PC-T, cpm/µg
PC efflux, cpm/µg cell protein
80
60
40
20
0
ApoAI 2 µg
ApoAI 5 µg
HDL
ME
ApoAI 2 µg
ApoAI 5µg
HDL
ME
3 h
24 h

Figure V.1

Figure V.2. The effect of trypsin treatment on the lipid efflux from smooth muscle cells. Rat vascular smooth muscle cells were loaded with radiolabeled cholesterol and choline-phospholipids as described. The cells were treated with 0.4% trypsin for indicated times. The cells were incubated with apoAI (10 μg/ml) or HDL (100 μg/ml) for 24 h. The lipids in medium and cell were extracted and separated. The background of 0.2% BSA was subtracted from the data. The left panel: apoAI-mediated lipid efflux; the right panel: HDL-mediated lipid efflux.

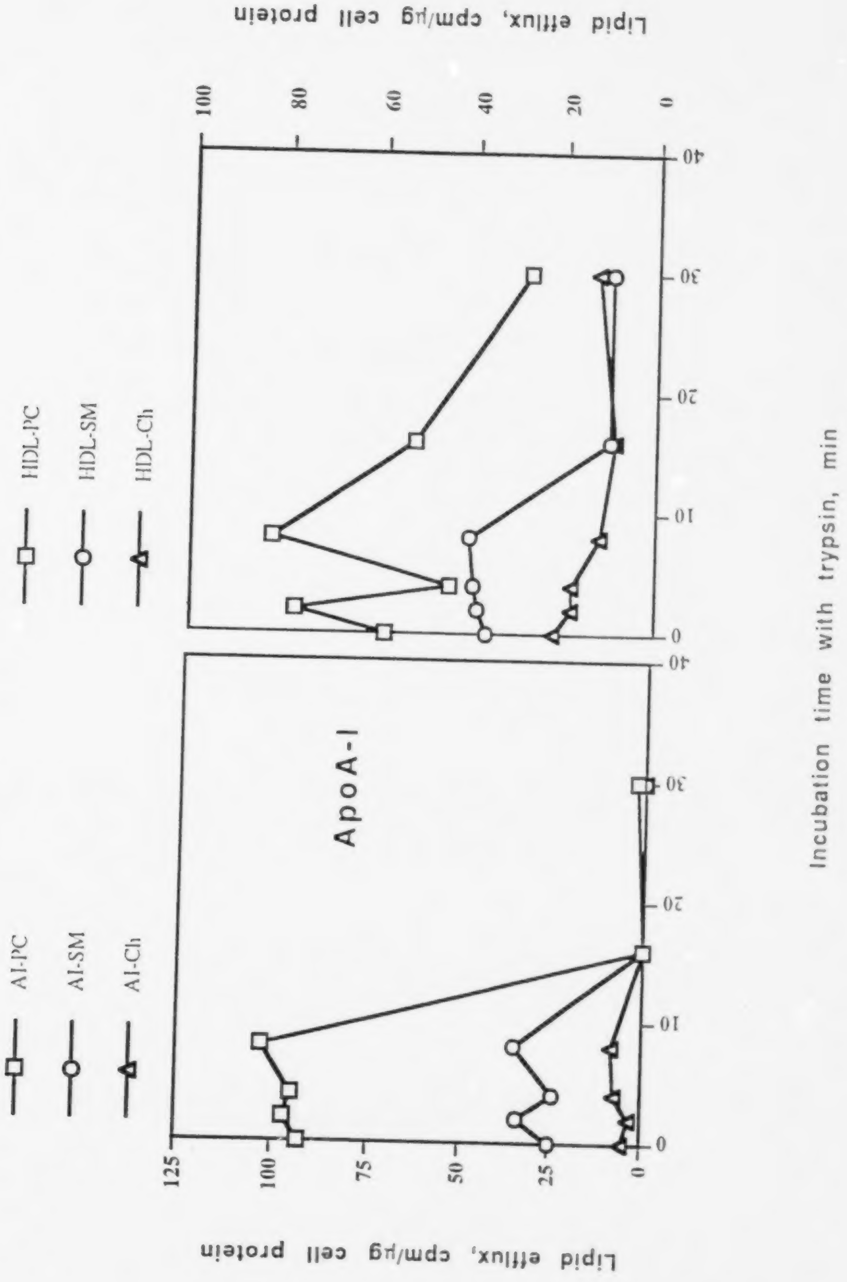

AI-PC
AI-SM
AI-Ch
HDL-PC
HDL-SM
HDL-Ch
ApoA-I
Lipid efflux, cpm/µg cell protein
125
100
75
50
25
0
100
80
60
40
20
0
0
10
20
30
40
Incubation time with trypsin, min

Figure V.2

Chapter VI.

DISCUSSION

In our lab, it was found that extracellular lipid-free apolipoproteins can interact with macrophages and mediate net cholesterol efflux by generating new HDL-like particle. The studies in this thesis are to investigate the mechanism of free apolipoprotein-mediated cholesterol efflux and the cellular specificity of this interaction. It is found that smooth muscle cells are resistant to free apolipoprotein-mediated cholesterol efflux. The experiments show that the resistance is not due to the resistance of smooth muscle cells to the interaction with apolipoprotein, but due to the poor availability of the cellular cholesterol for the reaction. It is also shown that this interaction of apolipoprotein with cells is a cellularly regulated process. The following is a summary and general conclusion of the project.

1. Cellular specificity of free apolipoprotein-mediated cholesterol efflux

In the previous study, it is found that apolipoproteins (apoA-I, apoA-II, and apoE) can mediate cholesterol efflux from cholesterol-loaded mouse peritoneal macrophages. This reaction has the following characteristics: (1) cholesterol efflux from cells is net cholesterol efflux; (2) the reaction requires very low concentration of apolipoproteins. Therefore, it is assumed that the small amount of free apolipoproteins dissociated from the HDL surface interact with the cell membrane and generate HDL-like particles. This mechanism contributes to the overall lipid efflux mediated by HDL. This process may play an important role in prevention of atherosclerosis and the formation of new HDL particles. To investigate the specificity of this reaction in certain types of cells that comprise of atherosclerotic lesion, we used vascular smooth muscle cells, fibroblasts, and macrophages to examine apolipoprotein-mediated cholesterol efflux (Komaba, et al. 1992). It was found that vascular smooth muscle cells are specifically resistant to this free apolipoprotein-mediated cholesterol efflux. In this reaction, for macrophages and fibroblasts, Vmax of apoA-I and apoA-II was about 30% that of HDL-Vmax, while for smooth muscle cells, Vmax was only 1-8% of the HDL-Vmax. Km of this reaction in smooth muscle cells is lower (1 μg/ml) than that of macrophages (5 μg/ml). This suggests that the resistance of smooth muscle cells to this reaction is not due to the lower reactivity of smooth muscle cells to the apolipoprotein, but due to the lower availability of cholesterol for this reaction. The observations may have the physiological relevance. In the advanced stage of atherosclerosis, the lesions are very resistant to regression because the major cell component in the lesions is smooth muscle cells which are resistant to free apolipoprotein-mediated cholesterol efflux. The understanding of the mechanism of smooth muscle cell resistance may give some hint for clinical therapy.

2. Mechanism of smooth muscle cell resistance to apolipoprotein-mediated cholesterol efflux

To understand the mechanism of this resistance, cholesterol efflux was examined in relation to phospholipid efflux from macrophages and smooth muscle cells (Li, et al. 1993). Lipid microemulsion is used to determine the lipid efflux through the pure physicochemical mechanism (nonspecific pathway); and LDL is used as a relatively nonspecific acceptor. The results are compared with those obtained in HDL and free apoA-I-mediated lipid efflux. It is demonstrated that the kinetic profiles of microemulsion-mediated cholesterol and phospholipid efflux from both smooth muscle cells and macrophages are similar. The ratio of phospholipid/cholesterol in LDL-mediated lipid efflux from both cells is similar as well. Therefore, it is concluded that the lipid efflux by nonspecific mechanism is essentially the same between both cells. In contrast, there are striking differences in apoA-I-mediated lipid efflux from both cells. The cholesterol efflux from smooth muscle cells is much lower than that from macrophages, while the phospholipid efflux from both cell is very similar. As a result, the phospholipid/cholesterol ratio of the lipid efflux from macrophages is lower (about 1) than that in smooth muscle cell (3-4). It is consistent with the conclusion that the resistance is not caused by poor reaction between smooth muscle cells and apolipoprotein but by poor availability of cholesterol. The phospholipid/cholesterol ratio is higher in HDL-mediated lipid efflux from smooth muscle cells than that of macrophages. This indicates the possibility that free apolipoprotein-mediated lipid efflux can contribute to the overall lipid efflux of HDL. The possibility is further confirmed by the finding that apoA-I significantly increases the ratio of phospholipid to cholesterol in microemulsion-mediated lipid efflux when excess of apoA-I is added to the microemulsion incubation medium.

3. Study of the cellular factor involving apolipoprotein-mediated lipid efflux.

The effect of HDL-binding protein in cholesterol efflux has been reported. This protein is up-regulated by loading cholesterol to cells. Upon HDL binding with the protein, cholesterol is reportedly translocated from the intracellular pool to plasma membrane pool. To examine the function of this type of the interaction in apolipoprotein-mediated cholesterol efflux, trypsin was used to hydrolyze cellular surface proteins. The treatment almost completely eliminated apoA-I-mediated lipid efflux but suppresses HDL- and microemulsion-mediated lipid efflux by only 25-50%. It suggests: (1) cellular integrity is required for the apolipoprotein-mediated lipid efflux; (2) the mechanism of apolipoprotein and HDL-mediated lipid efflux is different.

4. The effect of growth factors and phorbol ester on cholesterol efflux.

It is known that PDGF can stimulate SMC to express MCSF-receptor and other macrophage-like functions. As a result, smooth muscle cells are more active in migration and phagocytosis, and expected to be more responsive to apoA-I-mediated cholesterol efflux. Alternatively, PMA stimulates the PKC pathway and reportedly promotes cholesterol translocation from an intracellular pool to the plasma membrane pool. In our experiments, smooth muscle cell is treated with MCSF, PDGF and PMA to study the cholesterol efflux in macrophage-like smooth muscle cells. Results show that the treatment enhances cholesterol efflux from smooth muscle cells greatly without affecting phospholipid efflux. This enhancement of cholesterol efflux is only observed in apoA-I-mediated cholesterol efflux but not in HDL-mediated cholesterol efflux. It suggests that the treatment specifically affects on apolipoprotein-mediated cholesterol efflux. This indicates that the apolipoprotein-mediated cholesterol efflux is a cellularly regulated process but HDL-mediated cholesterol efflux is not totally regulated by a cellular mechanism.

The mechanism of this phenomenon is not clear yet. In order to understand the mechanism, many investigations should be done such as the measurement of cholesterol content of the plasma membrane, regulation of PKC activity, cellular cholesterol metabolism (cholesterol ester hydrolysis and cholesterol esterification) and cholesterol translocation.

REFERENCES

Hara, H. and Yokoyama, S. Interaction of free apolipoprotein with macrophages. J. Biol. Chem. 1991; 266: 3080-3086.

Komaba, A., Li, Q., Hara, H. and Yokoyama, S. Resistance of smooth muscle cell to assembly of high density lipoproteins with extracellular free apolipoprotein and to reduction of intracellular accumulation of cholesterol. J. Biol. Chem. 1992; 267: 17560-17566.

Li, Q., Komaba, A. and Yokoyama, S. Cholesterol is poorly available for apolipoprotein-mediated cellular lipid efflux from smooth muscle cells. Biochemistry. 1993; 32: 4597-4603.

Oram, J. F., Mendez, A. J., Slotte, J. P. and Johnson, T. F. High density lipoprotein apolipoprotein mediated removal of sterol from intracellular pool but not from plasma membrane of cholesterol-loaded fibroblasts. Arterioscl. Thromb. 1991; 11: 403-414.